METHODS
IN
MEDICAL RESEARCH

Volume 5 · A. C. CORCORAN, *Editor-in-Chief*

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Volume 5

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Volume 5

A. C. CORCORAN, *Editor-in-Chief*

METHODS FOR SEPARATION OF COMPLEX MIXTURES AND HIGHER MOLECULAR WEIGHT SUBSTANCES, *Lyman C. Craig, Editor*

METHODS OF RENAL STUDY, *A. C. Corcoran, Editor*

IMMUNOCHEMICAL METHODS OF DETERMINING HOMOGENEITY OF PROTEINS AND POLYSACCHARIDES, *Melvin Cohn, Editor*

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PREFACE

THIS SERIES of volumes has now, we feel, become known and valued to the extent that it is no longer necessary either to defend its institution or to explain its purpose and plan. Dr. A. C. Corcoran, who has been informally associated with the project from the beginning, now appears both as an Associate Editor and as Editor-in-Chief, and has furnished this volume with all the preface required. To him, to the other Associate Editors and the many contributors, we wish to express warmest thanks both on our own account and on behalf of the readers and users of the work. We are also indebted to readers of earlier volumes, and to other friends, for many helpful suggestions.

It is with regret that the Governing Board accepts the resignation of Dr. C. M. MacLeod, whose other duties have pressed too heavily upon him.

Irvine H. Page
A. C. Ivy
Colin M. MacLeod
Carl F. Schmidt
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EDITOR'S PREFACE

The editorship of a volume of *Methods* carries a somewhat equivocal status. The only firmly established tradition is that the editor should furnish an introduction, even when it is his impression that it will not be read by most who use the book. The introduction has two major purposes: one, it should provide some background on the collation of the volume and, two, it should enable the Editor to justify his share of the task.

At the outset, the Editor is guided in the selection of topics and the choice of Associate Editors by the Governing Board; the Associate Editors then order, arrange and edit their sections more or less autonomously. As a result, the Editor's task and responsibilities are far from overwhelming. Dr. Cohn's section on immunochemistry illustrates this principle of desuetude. For various reasons it was so delayed in transit that the Editor's task was wholly undertaken by Dr. MacLeod, a member of the Governing Board. To him our thanks and praise, as also the reader's, for the material lies within his special competence and the outcome therefore more fortunate than the plan.

Dr. Cohn's section also demonstrates a major function of the *Methods* series. Those who seek acquaintance with immunologic methods will appreciate the scope and importance of its content; others, more familiar with the field, will recognize that this body of information could only otherwise be obtained by visits to the laboratories of the contributors or by tedious and often unsatisfying searches of sources not readily available.

Dr. Craig's section illustrates other aspects of modern scientific technology. The methods apply to a wide variety of molecular species and indissolubly embody both physical and chemical principles. It can be fairly presumed that the techniques described will be as useful in industrial as they are in medical and biologic research. Thus, it seems that both the disciplines and the orientations of science have begun to converge.

The section brought together by the Editor's alter ego, Associate Editor Corcoran, is certainly the most heterogeneous and least penetrating of the three. The procedures listed range from ultramicroscopic anatomy to routine clinical testing. This variety is imposed by the topic, which is substantively the kidney as an organ for study rather than as an isolatable aspect of humoral biology. Since the procedures of renal research are usually adapted or designed for purposes of a particular experiment, many of those described will serve more as exemplars than as models.

Lastly, the Editor wishes to thank all who have contributed in any way and to join with Associate Editors and contributors in the hope that this volume—which is a tool, not a text—will find its place in laboratories rather than libraries.

—A. C. Corcoran.

CONTRIBUTORS AND REVIEWERS

BEYER, KARL H., M.D., Ph.D.
Assistant Director of Research and Chief of Pharmacology Section, Research Division, Sharp and Dohme, Inc., West Point, Pa.

BRADLEY, STANLEY E., M.D.
Associate Professor of Medicine, College of Physicians and Surgeons, Columbia University, New York.

BRODSKY, WILLIAM A., M.D.
Institute for Medical Research, University of Louisville School of Medicine, Louisville, Ky.

CÁMARA, AUGUSTO A., M.D.
Fellow of the John Simon Guggenheim Memorial Foundation, and Research Associate, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor.

CLARK, J. K., M.D.
Hospital of the University of Pennsylvania, Philadelphia.

COHN, MELVIN, Ph.D.
National Research Council Merck Fellow, 1949–52; Institut Pasteur, Paris, France.

CORCORAN, A. C., M.D.
Assistant Director, Research Division, and Staff Member, Cleveland Clinic Foundation, Cleveland.

CRAIG, LYMAN C., Ph.D.
Member, The Rockefeller Institute for Medical Research, New York.

DALTON, ALBERT J., Ph.D.
Cytologist, National Cancer Institute, National Institutes of Health, Bethesda, Md.

DEANE, NORMAN, M.D.
Department of Physiology, New York University Bellevue Medical Center, New York.

DEUTSCH, HAROLD F., Ph.D.
Associate Professor of Biochemistry, Department of Physiological Chemistry, University of Wisconsin Medical School, Madison.

DICKER, S. E.
Pharmacological Laboratory, University College, London, England.

DOMINGUEZ, RAFAEL, M.D.
Director of Laboratories, St. Luke's Hospital, Cleveland.

EARLE, DAVID P., Jr., M.D.
Associate Professor of Medicine, New York University College of Medicine, New York.

EDELMAN, I. S., M.D.
Research Fellow in Surgery, Harvard Medical School, Boston.

FORSTER, ROY P., Ph.D.
Professor of Zoology, and Chairman Science Division, Dartmouth College Hanover, N. H.

FREED, S. CHARLES, M.D.
Harold Brunn Institute for Cardiovascular Research, Mount Zion Hospital, San Francisco.

FRIEDMAN, MEYER, M.D.
Director, Harold Brunn Institute for Cardiovascular Research, Mount Zion Hospital, San Francisco.

GAUNT, ROBERT, Ph.D.
Professor of Zoology, Syracuse University, Syracuse, N. Y.: present address, Ciba Pharmaceutical Products, Inc., Summit, N. J.

GREGG, DONALD E., M.D.
Chief, Department of Cardiorespiratory Diseases, Army Medical Service Graduate School, Army Medical Center, Washington, D. C.

GREGORY, J. DELAFIELD, Ph.D.
Research Associate, Boston General Hospital, Boston.

GROLLMAN, ARTHUR, M.D.
Professor of Experimental Medicine, Southwestern Medical School of the University of Texas, Dallas.

HAUSMANN, WERNER, Ph.D.
Assistant, The Rockefeller Institute for Medical Research, New York.

HEYMANN, WALTER, M.D.
Assistant Professor of Pediatrics, Western Reserve University School of Medicine, Cleveland.

KABAT, E. A., Ph.D.
Associate Professor of Bacteriology, College of Physicians and Surgeons, Columbia University, New York.

KNOWLTON, ABBIE I., M.D.
Assistant Professor of Medicine, College of Physicians and Surgeons, Columbia University, New York.

LATTA, HARRISON, M.D.
Assistant Professor of Pathology, Western Reserve University School of Medicine, Cleveland.

LEWIS, LENA A., Ph.D.
Staff Member, Research Division, Cleveland Clinic Foundation, Cleveland.

LIPPMAN, RICHARD W., M.D.
Research Associate, Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles.

LITTLE, J. MAXWELL, M.D.
Professor of Pharmacology and Associate Professor of Physiology, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, N. C.

LONGSWORTH, LEWIS G., Ph.D.
Member, The Rockefeller Institute for Medical Research, New York.

MacINNES, DUNCAN A., Ph.D.
Member, The Rockefeller Institute for Medical Research, New York.

MASSON, G. M. C., Ph.D.
Staff Member, Research Division, Cleveland Clinic Foundation, Cleveland.

MOSS, W. GLENN, Ph.D.
Assistant Professor of Physiology, University of Illinois College of Medicine, Chicago.

NICKEL, JAMES F., M.D.
Assistant in the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York.

OLMSTED, FREDERICK, B.A.
Assistant Staff Member, Research Division, Cleveland Clinic Foundation, Cleveland.

ONCLEY, JOHN LAWRENCE, Ph.D.
Professor of Chemistry, Harvard Medical School, Boston.

OUDIN, JACQUES, Docteur en Medecine, Docteur ès Sciences
Chef de laboratoire, Institut Pasteur, Paris, France.

PAPPENHEIMER, A. M., Jr., Ph.D.
Associate Professor of Microbiology, New York University College of Medicine, New York.

PERLMANN, GERTRUDE E., Ph.D.
Associate, The Rockefeller Institute for Medical Research, New York.

PICKELS, EDWARD G., Ph.D.
Specialized Instruments Company, Belmont, Calif.

PITTS, ROBERT F., M.D.
Professor of Physiology, Cornell University College of Medicine, New York.

RODBARD, SIMON, M.D., Ph.D.
Assistant Director, Cardiovascular Department, Michael Reese Hospital, Chicago.

ROBSON, JAMES S.
Department of Clinical Chemistry, University of Edinburgh, Edinburgh, Scotland.

SCHOCH, HENRY K., M.D.
Assistant Professor, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor.

SEEGAL, BEATRICE C., M.D.
Associate Professor of Bacteriology, College of Physicians and Surgeons, Columbia University, New York.

SELKURT, EWALD E., Ph.D.
Associate Professor of Physiology, Western Reserve University School of Medicine, Cleveland.

SHEDLOVSKY, THEODORE, Ph.D.
Associate Member, The Rockefeller Institute for Medical Research, New York.

SHIPLEY, ROBERT E., M.D.
Lilly Laboratory for Clinical Research, Indianapolis General Hospital, Indianapolis, Ind.

SIROTA, JONAS H., M.D.
Research Assistant in Medicine, Mount Sinai Hospital, New York.

STEPKA, WILLIAM, Ph.D.
Research Associate in Plant Physiology, University of Pennsylvania, Philadelphia.

TAGGART, JOHN V., M.D.
Welch Fellow in Internal Medicine, National Research Council; Assistant Professor of Medicine, College of Physicians and Surgeons, Columbia University, New York.

TURNER, LOUIS B., M.D.
Research Assistant in Medicine, Mount Sinai Hospital, New York; formerly Research Fellow in Experimental Medicine, Southwestern Medical School of the University of Texas, Dallas.

WESSON, LAURENCE G., Jr., M.D.
Assistant Professor of Physiology, New York University College of Medicine, New York.

WEST, CLARK D., M.D.
Children's Hospital Research Foundation Cincinnati.

WRIGHT, LEMUEL D., Ph.D.
Director of Microbiological Chemistry Research Division, Sharp and Dohme, Inc., West Point, Pa.

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GOVERNING BOARD NOTE

Owing to the length and special production problems of this volume, royalties are being applied to the costs in order to maintain the selling price at the lowest possible level. Royalties for all other volumes of the series are distributed among the Editors and Associate Editors.

SECTION 1

Methods for Separation of Complex Mixtures and Higher Molecular Weight Substances

ASSOCIATE EDITOR—*Lyman C. Craig*

INTRODUCTION

MEDICAL RESEARCH has always profited from advances in the understanding of the complex physicochemical systems found in living matter. Among the techniques needed, not the least important are those which permit the separation and recovery of crucial substances from the biochemical matrix, occurring as they often do in relatively small amounts.

A large number of special methods are available for the determination or isolation of specific substances, but among the general methods of organic chemistry, such as distillation or crystallization, many are not suitable, either because of the unstable nature of the biochemically interesting compounds or because of the initial complexity of the mixtures.

Extraction with solvents has always been one of the most useful general procedures. As countercurrent distribution, it has now been advanced to the position of a highly powerful and specific preparative and analytical approach which can be adapted to a wide variety of substances, especially those requiring mild conditions.

Similarly, adsorption techniques have long been among the most useful tools for separating substances of biochemical interest.

Paper chromatography is a new development in this field which has already firmly established itself as one of the outstanding advances in the biochemical methods of recent times.

When larger molecular species are involved electrophoresis and the ultracentrifuge have been accepted without question for more than a decade as necessary tools. Nonetheless, recent years have brought forth improvements in technique for both methods.

—J. Delafield Gregory and Lyman C. Craig.

COUNTERCURRENT DISTRIBUTION

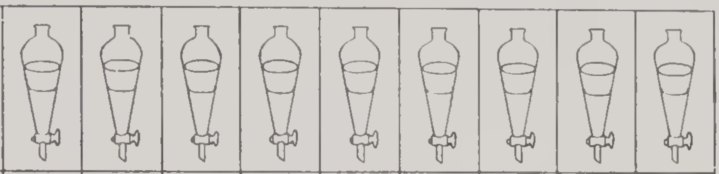
LYMAN C. CRAIG, *The Rockefeller Institute for Medical Research*

NEARLY EVERYONE working in the biological sciences as well as in chemistry is familiar with simple extraction as a tool generally applied in the first preliminary investigations of a new problem. It has been used this way for the separation of substances with widely differing properties since the beginning of chemistry. Only in recent years have its possibilities as well for the final characterization of a wide variety of closely related substances been really appreciated. Naturally realization of the fullest potentialities can be accomplished only through its integration with the many useful and specialized analytical techniques now available, and by rather extensive equipment. However, even with the techniques and equipment of most laboratories, far more can be accomplished than has been done in the past.

In dealing with an unknown preparation one of the first considerations should be that of fractionation. A mixture requires fractionation for isolation of the component of possible interest. A supposedly pure substance also requires a fractionation attempt in order to prove that it is not a mixture.

All methods of fractionation, in the broadest sense, have a common theoretical basis in that they deal with the manipulation of fractional parts. To be sure, in certain cases, the fraction removed or transferred is so large that for practical purposes separation is complete. The majority of separations, however, are not accomplished so easily and losses occur at each step, losses in labor as well as in material. Proper systematization minimizes such losses. This can be understood perhaps better by stepwise fractional extraction than by any other approach.

Let us take the case of a single solute, A, which can be distributed between 2 immiscible solvents. If at equilibrium with equal volumes of the 2 phases, half of the solute is found in each phase, then the partition ratio of the solute is 1. If the upper phase is transferred to a new equilibration tube (separatory funnel) containing an equal volume of fresh lower phase, and an equal volume of fresh upper phase is added to the first tube, the state of affairs represented by line 1, Table, of Figure 1, will be reached. Half the total solute will be in each of the 2 units. Now if both are equilibrated and the upper from 1 transferred to tube 2, that from tube 0 transferred to tube 1, fresh upper phase added to 0 and fresh lower phase added to tube 2, the state of affairs shown by line 2 will be reached: 0.25 of the original will be in tube 2, 0.5 will be in tube 1 and 0.25 will be in tube 0. If this process of alternate equilibration and transfer is continued until a total of 9 units is in the series, then the distribution of solute in each unit will be shown by the bottom line of the table. The number of stages applied is given by the vertical column on the left while the number of the unit is given by the top horizontal column.



	0	1	2	3	4	5	6	7	8
0	1.000								
1	.500	.500							
2	.250	.500	.250						
3	.125	.375	.375	.125					
4	.062	.250	.375	.250	.062				
5	.031	.156	.313	.313	.156	.031			
6	.015	.093	.234	.313	.234	.093	.015		
7	.008	.054	.164	.274	.274	.164	.054	.008	
8	.004	.031	.109	.219	.274	.219	.109	.031	.004

$(x+y)^n$

Let x = Fraction in upper layer

Let y = Fraction in lower layer

$$N = n\left(\frac{K}{K+1}\right)$$

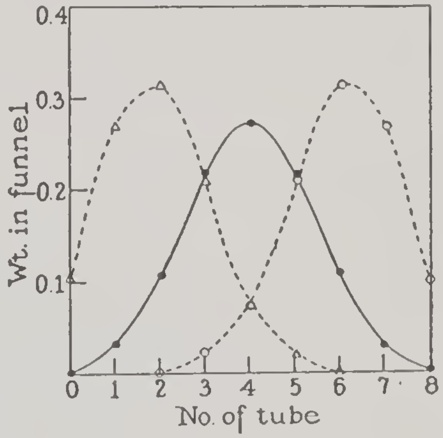


FIG. 1.—Distribution series; 8 stages.

If the fraction of the original in a unit is plotted against the consecutive number of the unit as given in the table, a distribution curve can be obtained with a maximum at tube 4. Had the partition ratio been 3, then the distribution would have been the curve on the right for 8 stages, while for a ratio of 0.333, it would have been that on the left. If 2 solutes had been present in equal amount and the K's had been 3 and 0.333, respectively, then the separation in each tube would be plainly evident from the 2 curves.

Mathematically, the Table of Figure 1 is represented by the binomial expansion, $(X + Y)^n = 1$, where n is the number of stages or *transfers*, X is the fraction in the upper phase and Y is the fraction in the lower phase. If X is the fraction in the upper phase of a single tube, then $1 - X$ is the fraction in the lower phase. By definition, the partition ratio K is $X/(1 - X)$. Thus X is equal to $K/(K + 1)$ and the fraction in the lower phase is $1 - [K/(K + 1)]$ or $1/(1 + K)$. The binomial expansion in terms of the partition ratio for equal volumes becomes

$$\left(\frac{K}{K + 1} + \frac{K}{1 + K}\right)^n = 1 \tag{1}$$

Obviously it soon becomes laborious to perform such multiple extractions individually in separatory funnels as represented. Nonetheless many laboratories have performed separations involving up to 20 or more separatory funnels in the series. Fortunately, mechanical equipment has been developed (10, 12, 20, 30, 16, 29) for the purpose and the number of units in the series can now be expanded almost indefinitely if the particular fractionation problem requires it. One extractor in use at the Rockefeller Institute (10) contains 220 equilibration units. It is fully automatic and is equipped with electric motors, time clock, etc. From 100,000 to 200,000 individual extractions can be performed in a 24 hr period. This equipment is commercially available.*

Such mechanical equipment, however, is valueless unless suitable systems can be found. The term system as used here means any mixture of liquids or solids which will yield 2 clear liquid phases when they are brought together and thoroughly mixed. The 2 phases are always equilibrated at the temperature of the experiment just prior to their use. To be suitable, a system must meet the following main requirements.

1. It must be capable of dissolving the solute of interest and should furnish a partition ratio within a practical range. If equal volumes of each phase are to be used in the mechanically operated equipment, the range would be from 0.01 to 100. With hand-operated equipment the range should be much narrower, from 0.2 to 5. In the latter case, however, compensation can be made easily for a K too high or too low by using a larger or smaller volume in either the upper or lower phase. Thus where r_v is the ratio of the 2 volumes, the product Kr_v should be within the limits of 0.2–5.

To reach the best separation possible with the least number of transfers, with a mixture of 2 components, the geometric mean of the 2 products, Kr_v and $K'r_v$ should be 1 (6). Here K and K' are the respective partition ratios of the 2 components.

---

* Obtainable from O. Post, 6822-60th Road, Maspeth, N. Y.

2. The partition ratio must be reasonably independent of the concentration. All solutes and systems show more or less deviation of K with concentration, particularly at higher concentrations. The adherence to ideality required will vary with the number of transfers applied. Thus a variation of 10 per cent over a 100-fold concentration range is not serious if no more than 20–30 transfers are to be applied. But, when hundreds of transfers are involved, closer adherence is desirable.

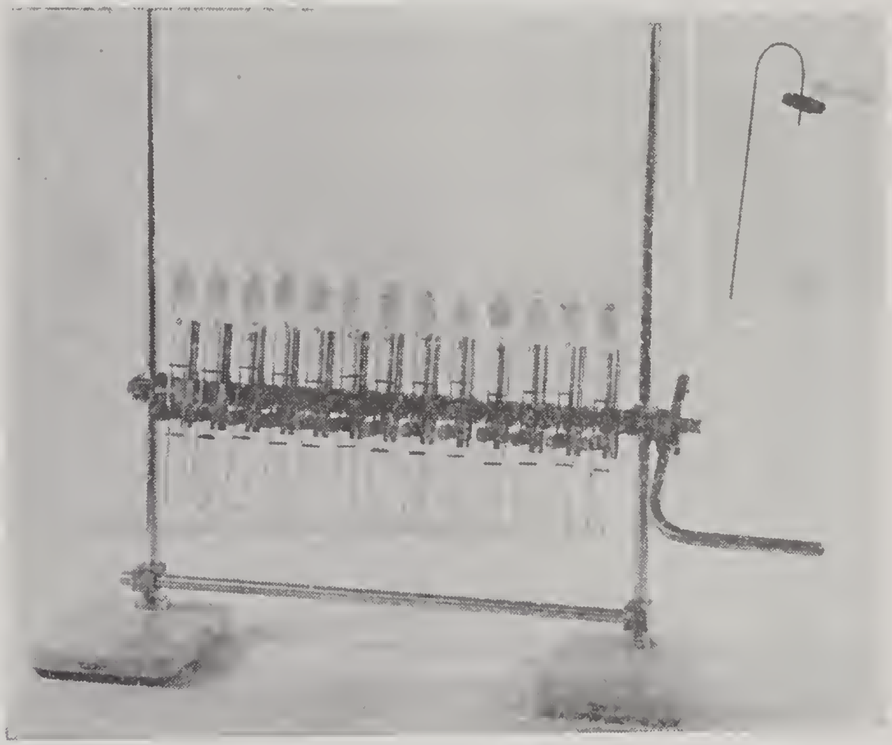


Fig. 2.—Test tube rack for preliminary countercurrent distribution.

In dealing with a new solute it is always worth taking the time, before launching forth on a countercurrent distribution run, to determine the K at the maximum concentration initially to be used in the run and at $^1/_{10}$ this concentration.

3. The system must permit equilibrium to be reached rapidly. This point is easy to test experimentally (3) when the partition ratio is determined. In general, partially miscible solvents cause little trouble in this respect.

4. The phases must separate clearly within a few minutes after equilibration and must not form stable emulsions. If emulsions do tend to form, lowering the pH probably will help. Addition of a

trace of a salt often is helpful. Increasing the volume of the organic phase often promotes separation. On close examination it may be found that a small amount of a solid film is separating at the interface. Removal of this will promote separation.

5. The solute must not be unstable in the system.

6. The system must lend itself readily to quantitative solute analysis, preferably by direct weight determination and to convenient recovery of the solute after the distribution is finished.

7. The system should not be expensive, extremely toxic or corrosive to the equipment.

Other less important points could also be mentioned. A system deficient in 1 requisite may be so favorable in the others that it is worth using in spite of disadvantages.

APPARATUS

When a suitable system has been chosen a preliminary run can be made. It may be made in the distribution apparatus or preferably in a row of glass-stoppered test tubes supported on a rack (12) as shown in Figure 2. The apparatus of Figure 2 can be assembled from standard laboratory equipment.

The tubes are held by spring clips attached by a screw to a short piece of Flexaframe rod. The stainless steel Gee clips, available from scientific supply houses, may be conveniently adapted. The rods attached to the clips are in turn attached to a longer rod which extends through 2 bearings on each end of the rod. The bearings are simply Flexaframe clamps in which the screw remains loose. The bearings are supported by 2 stands. A crank is attached to 1 end of the rod for tumbling the tubes.

In this design each tube receives its equal portion of stationary phase initially. The sample to be distributed is placed in tube 0. An arbitrary portion of the other phase, previously equilibrated with the first, is then introduced and equilibrium is reached by inverting the tubes with the crank. Twenty-five inversions are usually sufficient (3). The glass stopper will stick sufficiently not to fall out, if it is given a slight turn when it is placed tightly in position. Small interchangeable stoppers are therefore more reliable than larger ones.

After the layers have separated, the phase to be moved is transferred to the adjoining tube, 1, by a small siphon (Fig. 2, inset) preferably made from stainless steel tubing. Pressing the rubber collar, through which the siphon extends, against the mouth of the tube permits actuation by either air pressure or vacuum. The glass tube to which the pressure or vacuum line is attached also passes through the rubber collar and has a hole at the bend. The operator's finger is placed over this hole, so that instant release of the vacuum or pressure is possible.

Following the first transfer, fresh mobile phase is added to 0 and an equilibration made. The mobile phase of 1 then goes to 2, that of 0 goes to 1, and fresh phase goes to 0. This procedure is continued until all the tubes of the series contain both phases; i.e., the fundamental operation is complete. A series of 12 tubes can be filled in this manner in approximately 1 hr.

Analysis of the solute content/ml in both phases in each tube is then made and the result is plotted as in Figure 1 except that weight or some analytical unit proportional to weight/ml is used as the ordinate. Two curves will be obtained, 1 for the upper phases and 1 for the lower. From the data the partition ratio in each tube can be calculated directly. With a single solute or mixture with quite similar partition ratios the determined K in each tube throughout the series will be constant and the upper and lower curves will differ only in height rather than in position.

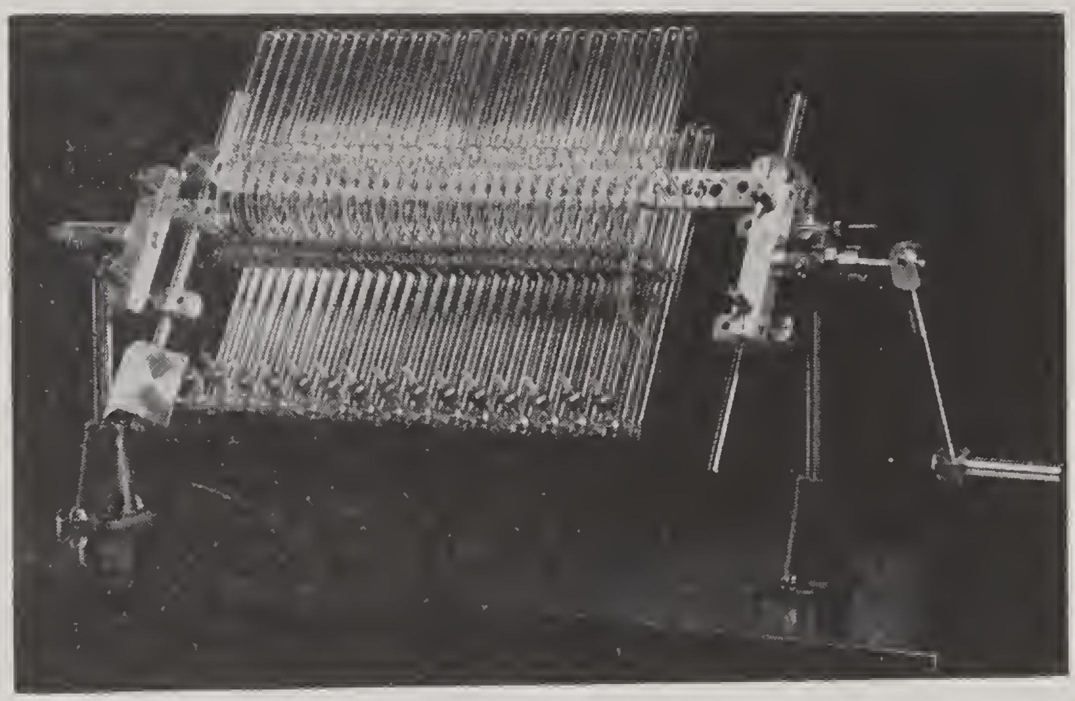


FIG. 3.—Thirty tube hand-operated train.

The pattern may reveal that a mixture has been sharply separated into 2 or 3 separate bands. More likely, however, a mixture will be revealed by a progressive increase in K from the lowest to the highest tube. Here a more extensive run with a machine is indicated. If so, the contents of each tube can be transferred to the corresponding tube of the machine in order to continue without making a fresh start.

The type of machine at present employed in the author's laboratory is actually a glass train (Fig. 3). The shorter trains with 25–100 units are hand-operated. A mechanical robot has been constructed for operating the longer trains. Figure 4 shows a 220 tube train equipped with such a robot.

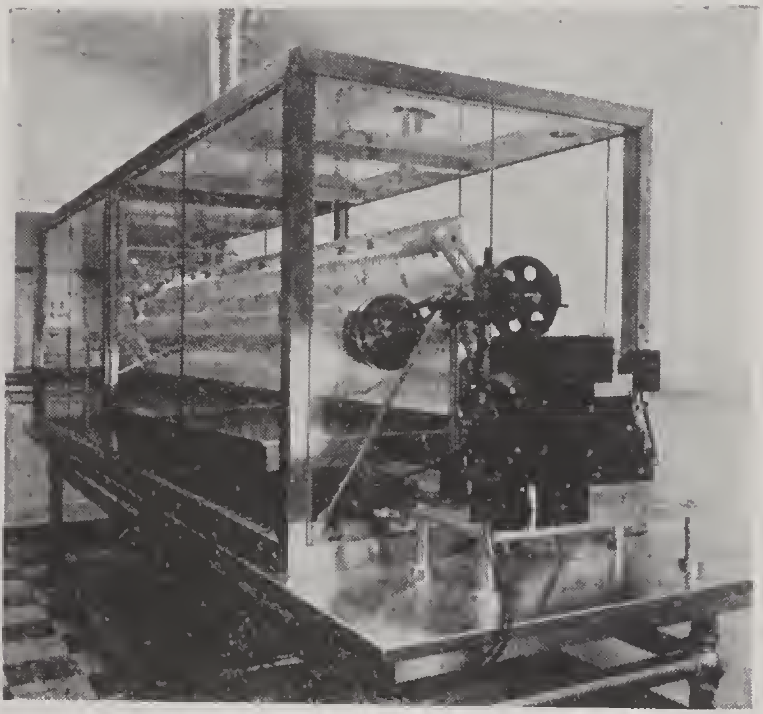


Fig. 4.—Automatic 220 tube apparatus.

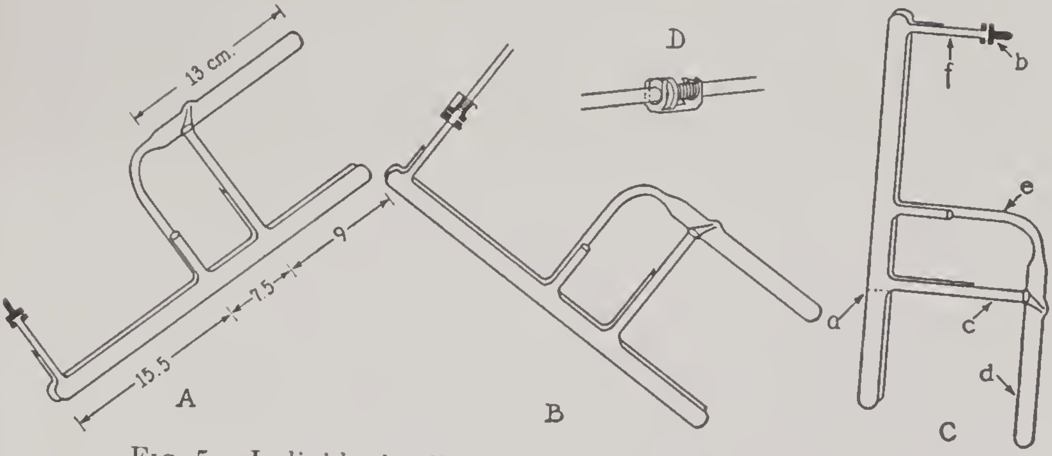


Fig. 5.—Individual cell construction of distribution train.

The construction of the individual tube or cell is shown in Figure 5. The cell is made from 15 mm glass tubing. It is approximately 32 cm long. The 3 tubes attached to it are from 7 mm glass tubing. The decantation tube *c* is 10 cm long. It passes through the lower wall of the decantation compartment *d* at a ring seal and extends nearly to the opposite wall. A shallow enlargement is blown in *d* opposite the opening of *c*. Like the cell, *d* is made from 15 mm tubing. Its opening at *e* is either sealed to the next equilibration cell of the series or is joined to it by a flat ground-glass stopper.

Access to each equilibration cell is permitted through *b*, which is a flat ground-glass stopper designed to close the tube *f*. The opening tube *f* is 4 cm long on the present apparatus. However, experience would now seem to suggest that a length of 8 cm would be preferable. The stopper *b* is held in position by a simple spring clamp *D*. Any device for opening and closing *f* must be simple and extremely rapid. Otherwise with 220 tubes in the series excessive time is required for the opening or closing operation.

The 2 phases in the equilibration cell are brought to equilibrium by rocking from position *A* to *B* and back again. Each position is about 40° from the horizontal. After a predetermined number of strokes, 5–50, depending on the rate at which equilibrium is established (3), the phases are permitted to separate at position *B*. Upon tilting further to position *C*, the upper phase decants through *c* to *d*. The length of the lower part of the cell is adjusted at the time of construction so that with a 10 ml lower phase, the meniscus separating the 2 phases only reaches point *a*. When the cell is tilted back to position *A*, the contents of *d* flow through *e* into the next adjoining cell of the series.

The cells are supported on two 4 × 4 cm duraluminum bars, each 180 cm long (Fig. 4). Two rows of cells are thus provided. The upper row is as close to the lower row as the height of the decantation tube will permit, but the former is placed slightly to the rear so that the stoppers closing the lower tubes are readily accessible.

The cells are held on the metal bar by means of a thin metal strap of stainless steel which passes over the glass tube and presses it against the duraluminum bar by a small bolt. The bolt passes through the bar to a nut on the under side. Notches have been cut in the bar so spaced as to receive each cell and hold it in position.

In the apparatus currently in use all the cells are sealed together through each overflow tube, *e*, except every 10th cell. At this interval they are joined by flat glass joints. The cells can therefore be handled and removed from the bar in banks of 10. Less opportunity for leakage is thus presented and it is necessary to use only 2 metal straps for the support of the 10 tubes to the duraluminum bar. On more recent commercial models each cell carries a joint and can easily be replaced in case of breakage. For a more complete description of the apparatus see reference (10).

In studies with the distribution machine thus far, 5 different methods of operation have proved most useful: (1) fundamental, (2) single withdrawal, (3) alternate withdrawal, (4) completion of squares, and (5) recycling.

FUNDAMENTAL PROCEDURE

This procedure must always be employed even when 1 or more of the other procedures is to be used later. The last 4 procedures are thus supplementary and permit a higher order of separation to be achieved without increasing the number of tubes to be manipulated in the series.

In the fundamental procedure, somewhat in excess of the amount of lower phase required for the entire train is inserted into the apparatus. The excess solution flows along as the run develops and finally emerges at the last tube.

After tipping the train to the decantation position a number of times, the solute to be distributed is inserted into the first tube with the upper phase (or into a small number of the first tubes). Several portions of upper phase are then placed in the tubes in front of the solute. These condition the lower phases before the solute reaches them.

A transfer is then accomplished and a fresh portion of upper phase is inserted at the 0 tube. The filling is made automatically if a filling device (10) is provided. The process is repeated until all the tubes contain their complement of upper and lower phase. Analysis is then made and a distribution pattern is plotted. This completes the fundamental procedure.

In case the pattern shows insufficient separation, an intelligent choice of one of the supplementary procedures can be made. Here the use of theoretical curves for comparison to those of the experimental will be helpful. The calculation of such curves will be taken up later.

SINGLE WITHDRAWAL

If a spectrum of partially separated bands is revealed by the fundamental pattern, the run can be continued by permitting the last upper phase to flow from the series at each transfer. Here the outgoing phase is assigned the transfer number which caused it to emerge from the series. It is set aside to be analyzed. The fraction collector of the automatic apparatus (10) takes care of the effluent phases automatically. The series emerging is analogous to that of effluent chromatography.

After an arbitrary time the run is interrupted for analysis again. A sufficient number of the tubes of the train and of the effluent series are analyzed so that distribution patterns can be plotted.

The pattern for the effluent series is placed to the right of the fundamental series with the highest transfer number adjacent to the highest tube number of the fundamental series. The abscissa for the effluent series is in terms of the transfer numbers which have been assigned to the effluent fractions. Again the pattern obtained is interpreted by the use of theoretical curves.

ALTERNATE WITHDRAWAL

In case the fundamental pattern shows that material in 1 or more of the earlier tubes of the series has been adequately fractionated, the contents of these tubes can be withdrawn and replaced by fresh solvent. Withdrawal can be made by removing a whole bank of tubes at 1 time or by removing a tube on every 2d, 3d, 4th, etc., transfer. Selected tubes are likewise removed from the other end of the series which contain the most rapidly moving solute. Two effluent series and a fundamental series thus can be obtained. One effluent series contains solute with lower K while the other contains solute with the highest K. The fundamental pattern contains solute with intermediate partition ratios. In plotting the patterns, the result for the material of lowest K is placed on the left of the fundamental pattern; that for the higher K on the right. Alternate withdrawal is most conveniently carried out with a circular apparatus (described in reference (12)) or in the glass train (described in reference (10)). A more complete description of the procedure is given in reference (12).

COMPLETION OF SQUARES

In this procedure, which is a systematization devised by Bush and Densen (6), the fundamental series is completed and the distribution is then continued without addition of upper phase. The effluent upper phases are collected as in single withdrawal. When the last upper phase has left the train 2 distribution patterns are constructed from the analytical data obtained from the effluent and the lower phases remaining in the train. The data are plotted in the same manner as in single withdrawal except that the pattern of the fundamental series is replaced by that of the lower phases. The tube numbers which were the abscissa of the fundamental pattern are therefore replaced by transfer numbers, with assignment to each tube of the transfer number which caused the upper phase to migrate from it.

RECYCLING

At the point of completion of the fundamental pattern, 2 or 3 overlapping bands may be revealed with no solute in either or both extremities of the pattern. More transfers may be applied by reintroducing the effluent upper phase from the highest tube as the upper phase required at tube 0. This can be continued until the front of the advancing solute band almost catches up with the trailing edge. In this way many more transfers may be applied and a much better separation achieved without greatly increasing the total volume of solvent.

Such a procedure becomes most useful when a considerable number of units or tubes is in the series. For example, with a single pure solute being distributed in a train of 25 tubes, the advancing solute front would already be overtaking the trailing rear at 60 transfers. On the other hand, if 220 tubes are in the series the advancing solute front would not be overtaking the trailing edge until more than 5,000 transfers had been applied.

To take full advantage of this fortunate circumstance, the automatic 220 tube apparatus (Fig. 4) is arranged so that it really is a double train containing 110 cells in each series. In the lower series the upper phases flow in the opposite direction to those in the upper series. Thus tube 219 is directly under tube 0 and the upper effluent phase can be diverted automatically into 0 again if this is desired.

In this procedure the results are plotted as in the fundamental case provided solute has not migrated beyond tube 219. Where it has re-entered the series for a second circuit, tube 0 becomes 219 + 1 or tube 220. Tube 1 becomes 221, etc.

Calculation of theoretical curves.—A single pure solute behaving ideally in a given system will give a characteristic and reproducible distribution of mass throughout the series of cells. Since this distribution will depend only on the number of transfers applied and the partition ratio (or Kr_v where unequal volumes of the phases are used), the distribution to be expected can be calculated directly at any time. Comparison of the calculated curve with the experimental one then permits a much more critical evaluation of the question of purity or, if the pattern indicates overlapping bands, theoretical curves permit the most intelligent choice of one of the foregoing procedures in order to reach the degree of separation desired with the least trouble.

Numerous methods for calculating theoretical curves have appeared during the past few years (2, 8, 18, 19, 21, 25, 31). Obviously all of these cannot be treated here because of the limitations of space. Therefore only the ones most used in our laboratory will be treated.

From the binomial of equation (1), the fraction of the original single solute present in any one tube (in upper and lower phases combined) can be calculated. A general formula for this purpose (31) is found in equation (2) where

$$T_{n,r} = \frac{n!}{r!(n-r)!}\left(\frac{1}{K+1}\right)(K)^r \qquad (2)$$

is the fraction in the rth tube at n transfers. Rather than calculate each value independently by equation (2), the desired curve can be reached more rapidly by calculating a single term first by equation (2) and then by calculating the remainder from this term through the use of simple factors as given below.

In general the rth term is related to the $(r + 1)$th term by equation (3), where $F' = (r + 1)/(n - r)$. The F' factors are thus 1/8, 2/7, 3/6, ...8/1

$$T_{r+1} = T_r K/F' \qquad (3)$$

for calculation of the 1, 2, 3, ... 8 tubes from tube 0 in an 8 stage distribution. The fraction in tube 0 is $(1/K + 1))$. Similarly, the factors for a 10 stage distribution would be 1/10, 2/9, 3/8, ... 10/1.

When higher numbers of transfers are concerned and where the band has cleared both the 0 tube and the highest tube, a different method of calculation is preferred. The approximation in equation (4)

$$y = \frac{1}{\sqrt{2n\pi K/(K+1)^2}}\, e^{-x^2/[2nK/(K+1)^2]} \qquad (4)$$

which can be derived from the equation for the normal curve of error, permits a single curve for any number of transfers n to be calculated with the aid of a slide rule in 10 min.

In matching a theoretical curve to the experimental, the y_0 term of the distribution usually is assumed to be the maximum of the experimental curve. Since here x is 0, equation (4) simplifies to equation (5).

$$y_0 = \frac{1}{\sqrt{2n\pi K/(K+1)^2}} \qquad (5)$$

Following the assumption that y_0 is the experimental maximum, the next point of interest becomes the degree to which the other calculated points of the theoretical curve will match with the experimental. The calculated y values each are related to y_0 by the factor

$$e^{-x^2/[2nK/(K+1)^2]}$$

Therefore $2nK/(K + 1)^2$ can be considered as a constant appearing in the calculation of each Y value. The constant e also appears in each calculation. In calculating a y value x tubes removed from y_0

$$\frac{0.434}{2nK/(K+1)^2}$$

is the logarithm to be used as a factor in each calculation. Let this logarithm be L. The fraction of the original solute present in any tube x tubes removed from y_0 is therefore related to y_0 by the factor 1/antilog of x^2L. Calculation of approximately 5 of these factors is sufficient for a theoretical curve to be matched.

In the case of a *single withdrawal* series the mathematics are even less complicated than in the case of the fundamental series. Here the partition ratio can be calculated from equation (6)

$$K = u/(n - u) \qquad (6)$$

where u is the number of tubes in the train and n is the transfer number of the maximum. The theoretical curve can be calculated as given above for the fundamental series except that equation (7)

$$y = \frac{1}{\sqrt{2\pi n/K}} e^{-x^2/[2n/K]} \qquad (7)$$

must be used instead of equation (4).

Calculations for the *alternate withdrawal* series are much more complicated and will not be treated here. However, there is little difficulty in calculating curves for the *completion of squares* procedure or for the *recycling* procedure. The latter is identical with the calculation of the fundamental curve. Bush and Densen (6) have given excellent mathematics for calculating the former. Where many tubes have been used in the series, equation (7) can be employed for calculating the theoretical curve for the effluent upper phase. The lower phase pattern can be considered as the inverse of this. Therefore $1/K$ should be used in equation (7) for calculating the theoretical for the effluent lower phases. K in this case is obtained from equation (8)

$$K = s/(n - s) \qquad (8)$$

where s is the number of lower phases left without upper phases over them when the last upper phase leaves the lower phase of highest solute concentration.

DETERMINATION OF WEIGHT PATTERNS

The importance of weight patterns in fractionation procedures as contrasted to patterns obtained by other analytical methods is sufficient to warrant a short description of the procedure now in use in our laboratory.

a) *Apparatus.*—A series of thin glass shells serve both as evaporation and weighing vessels. They are made by blowing a round bulb, approximately 3.3 cm in diameter, from soft glass ampules. The lower half of the bulb is detached by scratching a line on the fragile wall with a diamond point and then cracking off the hemisphere with the hot wire of a glass cutter. The cracked edges are strengthened by fire polishing. A sufficient number are made so that shells weighing within ±50 mg of each other can be selected. The weight of a finished shell should approximate 0.5 g.

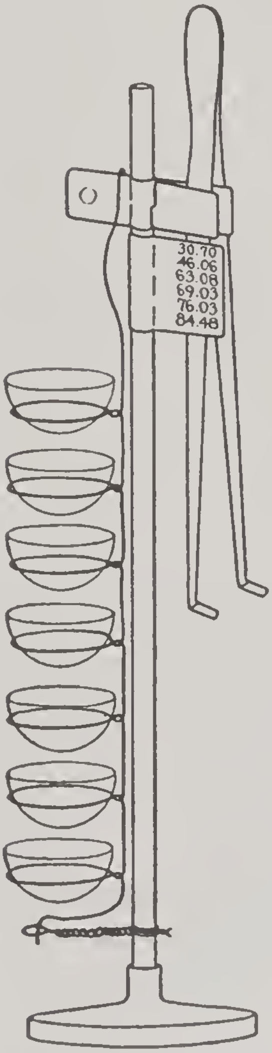


Fig. 6.—Glass shells for weight analysis.

The shells are arranged in order of increasing weight on a wire frame (Fig. 6) which can be made by twisting a series of loops from a copper wire. Loops of the proper size result from molding the wire around a glass tube of 3 cm OD. The wire frame is hung on a small tripod which also supports a pair of small forceps for manipulating the shells. The jaws of the forceps can be covered with small pieces of rubber tubing to avoid damaging the fragile shells.

The evaporator is a small steam bath placed on an electric hot plate. The cover of the steam bath is fashioned from a flat sheet of stainless steel with 5 holes drilled in it. One of the holes permits a reflux condenser to be attached, and the other 4 are of such size, 3.0 cm, that the glass shells will not fall through, yet large enough so that most of the under surface of the shells will be exposed to the hot vapors when the shells rest in the holes. A glass tube, 10 mm ID, with its opening approximately 2 cm above each shell serves as an air jet.

The top shell of the series is a control tare. If the solvent system itself contains a measurable residue, it may be advisable to evaporate an appropriate aliquot of the solvent as a blank each time a series of determinations is made. In this manner the weight of each fraction subsequently determined is automatically corrected. Otherwise, the tare only receives the same final drying treatment as the other members of the series.

b) *Technique.*—An aliquot of the solution to be analyzed, 0.1–3.0 ml, is added to the second shell after it has been placed on the steam bath. A hypodermic syringe is ideal for this purpose, and can be fitted with a fine glass tip drawn out from an adapter.† A current of air to the jet is filtered free of dust particles through a cotton plug and is blown at the solution with no more than sufficient speed to be barely noticeable on the surface of the solution.

If an aqueous solution is to be analyzed, it will require 2–3 min for a 1 ml aliquot to be brought to dryness at 100°. Higher or lower temperatures can be reached as desired by placing liquids of suitable boiling temperature in the bath. During the time required for the evaporation of the first aliquot, others can be started on the remaining 3 holes of the bath.

As soon as the solution comes to dryness, the liquid clinging to the bottom of the shell is touched off with cotton gauze, and the shell is placed in its proper position on the wire frame. When all of the shells have been so treated, the wire frame with its shells is placed in a large glass test tube fitted with a rubber collar near its upper end. The tube and its contents are supported in a steam bath by means of this collar, and the open end is closed by a 1-hole rubber stopper with a glass tube and rubber hose connection to a high vacuum oil pump. All the samples and the tare are dried together at 100°/0.2 mm for the desired time, usually about 5 min. The shells are then weighed on a semimicrobalance to ±0.01 mg. In weighing, the tare always counterbalances the shell containing the residue.

The Seederer-Kohlbusch balance used in this laboratory utilizes riders for weights up to 100 mg, and hundredths of a milligram are obtained by measurement of the degree of deflection. Since the shells do not differ from the tare by more than 100 mg. the entire series of shells can be weighed by use of the riders alone. The balance is magnetically damped, and each weighing requires no longer than $1^1/_2$ min.

Systems for separating various types of compounds.—The decision as to whether a particular system is the one most satisfactory for a given type of substance can be very time-consuming if the separation is likely to be difficult and if many different combinations of solvents are tested. Much time and effort have been spent along this line by various workers in this laboratory, perhaps considerably more time than has been devoted to the theoretical, mechanical and analytical aspects of the development of countercurrent distribution. It therefore appears highly advisable to mention some of the more satisfactory systems that have been used for various types of substances.

ORGANIC ACIDS.—For the lower fatty acids, hydroxy acids and dicarboxy acids, a system containing a concentrated buffer (4), such as phosphate, for the aqueous phase and a variety of organic

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† Available from Becton, Dickinson and Co., Rutherford, N. J.

solvents for the nonpolar phase has been most useful. The organic phase can be isopropyl ether, chloroform, benzene, heptane or an alcohol such as butanol. Isopropyl ether redistilled and preserved over iron wire to retard peroxide formation has been one of the most useful solvents for the organic phase. The desired value of partition ratio can be obtained by varying the pH and concentration of the buffer or by choosing an organic solvent with more hydrophilic properties where the *K* favors the aqueous phase.

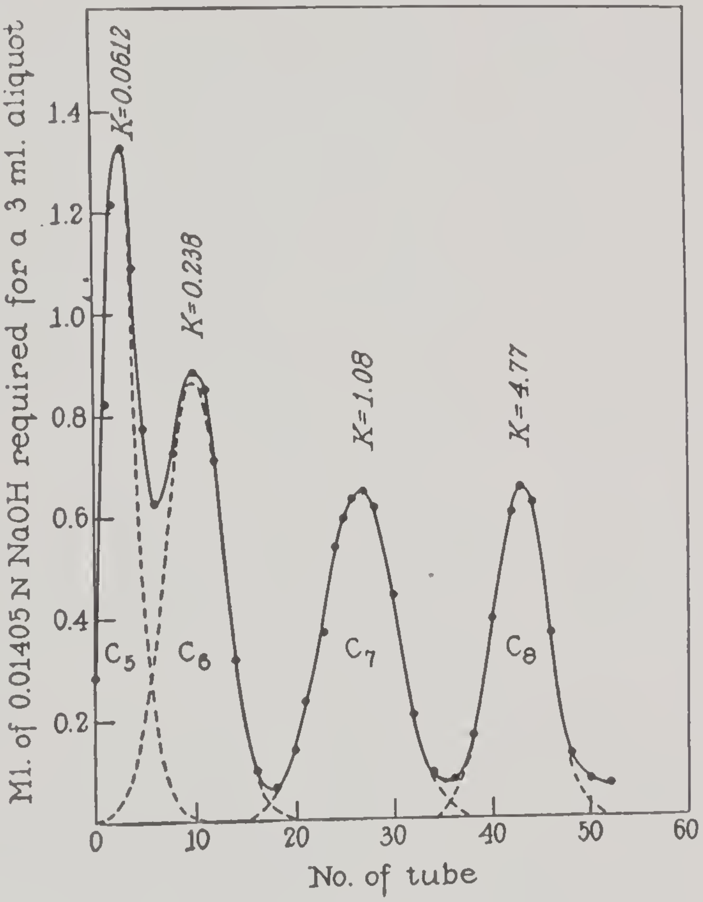

FIG. 7.—Separation of C_5 to C_8 acids.

The reverse is followed where the solute favors the organic phase. Mixtures of solvents can also be useful for reaching a satisfactory *K* value.

As an example, Figure 7 shows the separation of the normal C_5, C_6, C_7 and C_8 acids in 52 transfers. For the higher fatty acids, however, a buffered system did not prove suitable because of their poor hydrophilic properties and tendencies to give skewed patterns. Here a system made by equilibrating equal parts of n-heptane, glacial acetic acid and formamide appeared satisfactory. Figure 8 shows the separation obtained in 400 transfers with a mixture of 300 mg each of C_{12}, C_{14}, C_{16} and C_{18} acids (1).

ORGANIC BASES.—The type of system found most useful for bases is very similar to that most selective for the organic acids. In fact, buffered systems (9, 11, 14, 28) gave relatively much greater selectivity in separating basic mixtures. The method is ideally suited for the separation of complex mixtures of alkaloids (13). In the majority of cases the buffered systems are so selective that distributions involving high numbers of transfers are not at all necessary.

AMPHOTERIC SUBSTANCES.—In this class of substance much work remains to be done. Buffers appear to contribute little to selectivity. Incorporation of highly charged solutes such as salts into the system appears to give somewhat greater selectivity, but here the problem of elimination of the salt in order to permit weight analysis partially offsets the advantage. Ammonium acetate does not have a high salting out (or salting in) effect but can be eliminated by virtue of its volatility. It is therefore a valuable adjunct. If the substances are stable in acid they may be distributed as hydrochlorides by using dilute hydrochloric acid in the aqueous phase. Such a system is particularly valuable for separating amino acids from the hydrolysate of a peptide or protein. Table 1 gives K values for the common amino acids in a system made by equilibrating equal volumes of n-butanol and sec-butanol with 5 per cent

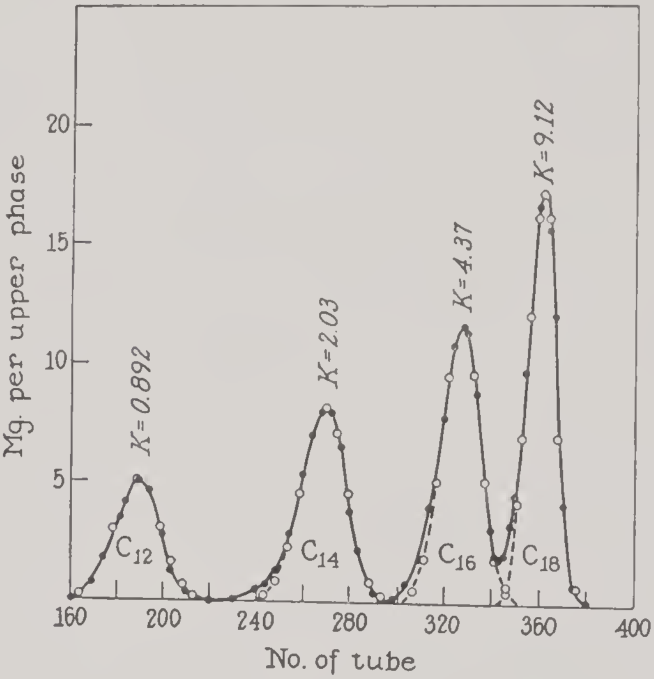

FIG. 8.—Separation of C_{12}, C_{14}, C_{16} and C_{18} acids.

TABLE 1.—Partition Ratios of Amino Acids

Ornithine·HCl	0.053	Aspartic acid	0.21
Lysine·HCl	0.058	Cysteine·HCl	0.24
Cystine	0.060	Glutamic acid	0.29
Histidine·HCl	0.065	α-Aminobutyric acid	0.303
Taurine	0.088	Valine	0.40
Arginine·HCl	0.089	Methionine	0.46
Glycine	0.103	Tyrosine	0.65
Serine	0.118	Iso-leucine	0.87
Alanine	0.15	Leucine	0.90
Hydroxyproline	0.15	Phenylalanine	0.92
Threonine	0.154	Tryptophan	1.87
Proline	0.18		

hydrochloric acid. Most of these values have been substantiated in actual runs (10).

Where bands overlap in this acid system they may be separated in a neutral n-butanol/water system or in an alkaline system. A system made by equilibrating a solution of 2 parts sec-butanol and 1 part propanol with an aqueous solution containing 10 per cent ammonium acetate and 3 per cent ammonia permitted the separation of phenylalanine, leucine, tyrosine, valine, proline and a band containing aspartic acid, glutamic acid and ornithine. The pattern shown in Figure 9 was obtained on a hydrolysate of tyrocidine A (5).

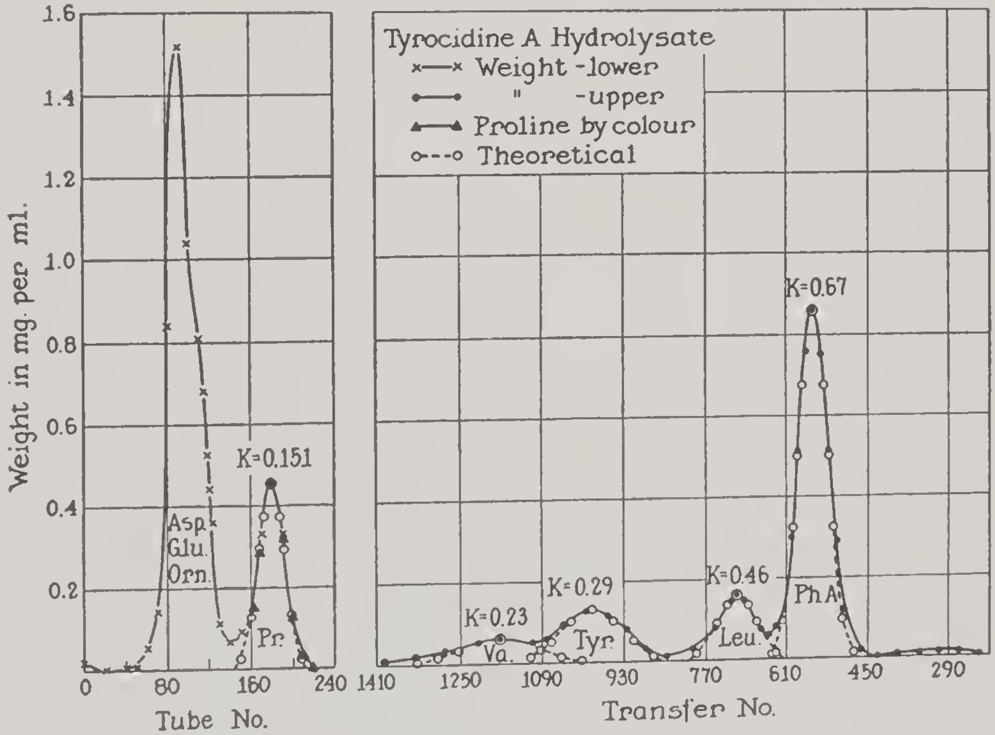

Fig. 9.—Separation of amino acids from tyrocidine A.

For the separation of the higher polypeptides a variety of systems have been developed. Table 2 gives a few of the systems most useful for the various classes of naturally occurring peptides thus far studied in our laboratory. Although insulin properly belongs to the protein class, it too has been brought under study and successfully distributed.

Substances which are so strongly hydrophilic that they are scarcely soluble in any organic phase can be complexed with a suitable complexing agent such as a detergent. These agents have been called carriers by O'Keefe *et al.* (23). This approach has been useful in developing systems for streptomycin (27), neomycin (26), heparin (22), nucleotides and phosphorylated sugars (24). The distribution of insulin (17) using di- and trichloracetic acid in the system probably also comes in this category.

TABLE 2.—SYSTEMS FOR PEPTIDES

Penicillins	Ethyl ether/phosphate buffers
Gramicidins	System made from water, methanol, chloroform and benzene in 7, 23, 15 and 15 vol proportions, respectively
Tyrocidines	Methanol, chloroform and 0.1N hydrochloric acid in 2, 2, 1 vol proportions, respectively
Gramicidin-S peptides	Methanol, chloroform and 0.01N hydrochloric acid in 2, 2, 1 vol proportions, respectively
Polymyxin type	Systems made with 2-butanol and 0.01–0.5N hydrochloric acid
Bacitracin	2-butanol/3% aqueous acetic acid or n-butanol/0.1M NaH_2PO_4
Insulin	2-butanol/1% aqueous dichloracetic acid

A number of interesting systems for separating sterols have been studied by Cornish *et al.* (7). Heptane and acetonitrile form a particularly good system. The chlorophyls can be separated readily in the system hexane/90 per cent methanol. Golumbic and coworkers (15) have given systems for separating phenolic compounds.

Protocol: Distribution of crude gramicidin-S hydrochloride. Since gramicidin-S was known to contain only basic groups it was thought best to distribute it in a system containing hydrochloric acid. This would maintain it as the hydrochloride. A preliminary survey of systems by determination of partition ratios in various mixtures suggested a system containing methanol, chloroform and 0.1N hydrochloric acid in the volume proportions 2, 2, 1.

The apparatus available for the work was an all glass train (12) containing 108 tubes. After combining the solvents and equilibrating at the temperature of the room, sufficient of the heavier phase (1,100 ml) to fill the lower phases in each tube was scattered in the first 15 or 20 of the tubes. The apparatus was then tipped to the decanting position about 15 times, thus clearing the first 15 tubes of excess lower phase and moving it on to empty tubes further along the train. Volumes of the lower phases are automatically adjusted in this way as the distribution proceeds.

Ten upper phases were then placed in tubes 3–13. These phases serve as a forerun to insure proper equilibration of the system. The sample 1.0 g, was placed in 30 ml of each phase and these solutions divided among tubes 0, 1 and 2.

Equilibration was accomplished by rocking back and forth 10 times. The phases were permitted to separate with the apparatus tipped halfway to the decantation position, then tipped farther so that all the upper phases decanted and then tipped back again to the transfer position. A fresh upper phase was added to the 0 tube, etc. After 106 transfers the effluent was collected in test tubes marked with the transfer number on which each fraction emerged from the train.

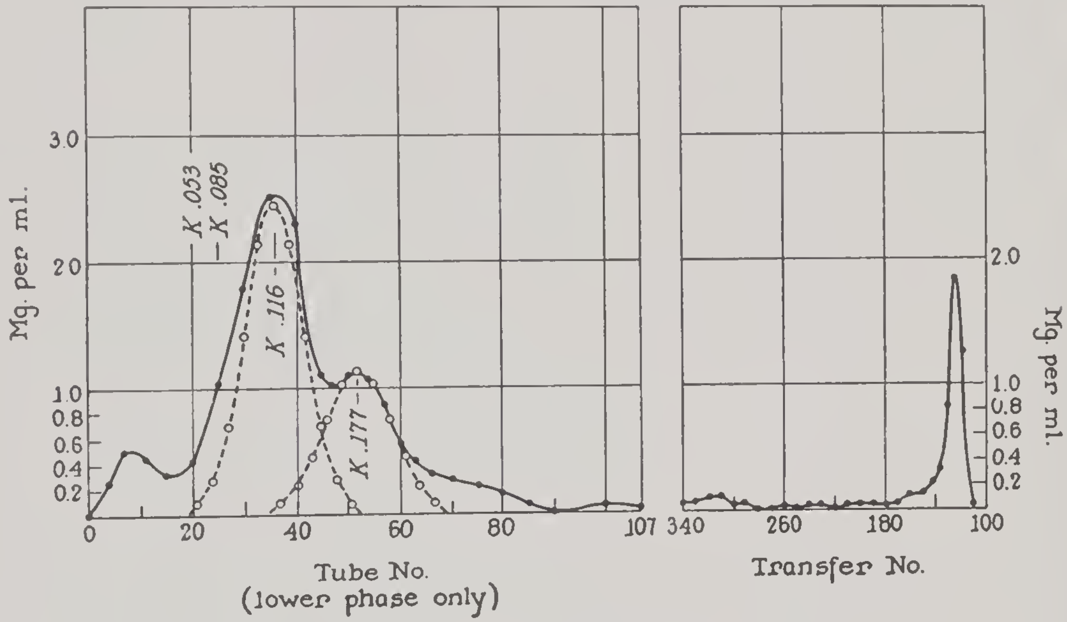

Fig. 10.—Distribution of gramicidin-S.

At 340 transfers the distribution was evaluated by weight analysis. Since at this point only solutes of low partition ratio remained in the train, only aliquots of the lower phases were examined. The weight/ml was plotted against tube numbers or transfer numbers, as shown in Figure 10. It is necessary to analyze only enough tubes to give the curve reliably, every 5th, 10th or 20th tube, depending on the width of a band.

Note.—This section was reviewed by J. Delafield Gregory.

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PAPER CHROMATOGRAPHY*

WILLIAM STEPKA, *University of Pennsylvania*

Two main features distinguish paper chromatography from classic methods of analysis. (1) Remarkable selectivity makes it ideally suited for analysis of multicomponent mixtures. With proper precautions unambiguous results frequently can be obtained even if the mixtures contain such closely related substances as homologues. (2) The possibility of performing a qualitative analysis on micro amounts of sample permits, for example, determination of amino acid residues from as little as 300 μg of protein (33, 94, 138). This possibility is often advantageous in other applications, notably in studies of intermediary metabolism with tracers. Here a radioautograph of a chromatogram not only shows the minimum number of radioactive compounds involved but also indicates the scope of the problem confronting the investigator. Frequently the probable nature and identity of the spots can be established with little extra effort. Such chromatographic information can be of great value in the task of ultimate isolation and characterization.

In many applications, such as the study of soluble fractions from tissues, paper chromatography allows analysis without introduction of reagents stronger than those used for development. For complex mixtures likely to contain labile components, the virtue of this is evident.

Introduction of filter paper into partition chromatography by Consden, Gordon and Martin (33) followed earlier work of Martin and Synge (97), who conceived the idea of separating components of a mixture by liquid-liquid partition in a countercurrent machine and in common with previous workers foresaw its general applicability. A search for another means of applying the same promising principles led to gradual extension of the concept by them (62, 63, 94, 98) and others (150). Preliminary trials with filter paper as a support led to the development by Consden *et al.* of the 2-way paper partition chromatogram (33). Description and use of this general analytical method constitute the major topics of this discussion.

Principles.—Stated briefly, paper chromatography is a technique for separating a mixture of substances into its components by allowing a solvent system to creep unidirectionally through a spot of the mixture on a paper support. Capillarity or capillarity aided by gravity causes movement in the predetermined direction.

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* Part of this section was written during the tenure of an AEC Predoctoral Fellowship at the University of California.

A single passage of the solvent through the spot produces a 1-way or "strip" chromatogram. When, however, the origin contains numerous substances a single excursion will rarely result in complete separation of the components. This ideal can be more nearly realized with the 2-way chromatogram. Here, the mixture, applied near 1 corner of a rectangular sheet, is developed initially as a 1-way chromatogram. Then by turning the sheet through 90°, the separations achieved in the first run become a series of parallel "origins" for development with another solvent. If properly chosen the second solvent may resolve the overlaps from the secondary origins. Such double development produces the 2-way chromatogram with its characteristic pattern of spots.

Consden *et al.* (33) regarded the separation of solutes as a function of their partition coefficients between 2 liquid phases. Accordingly, the paper is represented as an inert support which, in effect, immobilizes 1 member of the solvent pair (water), holding it stationary while the organic member slowly flows past in a thin liquid sheet.

With this concept the process responsible for separation can be imagined as follows: At any instant solute molecules at a given position on the paper become distributed between the stationary and mobile phases in accordance with their partition ratio in the 2 phases. Since the organic phase is moving and a continuous supply is available from the source, solute which passed into this phase at location A will be carried along to another but proximate position B. At B, however, no solute molecules are initially present in the underlying water phase, hence solute will pass from the organic phase into the water phase until the respective concentrations prevailing at B are again in accord with the distribution law. Thus, by continuous adjustments of distribution equilibria at successive sites, a given solute is transported from the area of origin to some other area along the path of the advancing phase. The distance through which any solute will move is governed by the ratio of its instantaneous concentrations in the 2 phases. Migration is favored by an increase of the fraction in the mobile phase, and conversely.

The high efficiency of partition chromatograms (98, 102) may be attributed largely to 2 factors: (1) availability in the paper of numerous sites at which adjustments of equilibria can occur; (2) the rapid establisbment of each equilibrium, since only minute distances need be involved in the transfer of solute between phases.

The ratio of the distance through which a compound travels to the distance through which the mobile phase has moved, measured from the point of application or origin, has been called the R_f value (33). Under given conditions of solute concentration, solvent pair, paper, pH and temperature, this value remains fairly constant for any one compound (33). Consequently it is possible to prepare chromatographic maps (33, 46, 110) showing the expected position of a given compound relative to other compounds (Fig. 1). The effects of variables are considered later; of interest here is the bearing R_f values have on the interpretation of the mechanism involved in the separation.

Consden *et al.* (33) derived partition coefficients for certain amino acids from their observed R_f values and compared them with those determined classically by England and Cohn (Table 1 in (33)). Close agreement led them to rule out adsorption phenomena and to explain movement and separation solely on principles of liquid-liquid partition. Synge (99, 143) working with amino acids from hydrolysates of gramicidin similarly supported this interpretation.

TABLE 1.—Comparison of Measured and Calculated R_f Values in Butanol-Propionic Acid-Water Solvent*

SOLUTE	MEASURED α	R_f	
		Calculated	Measured
Alanine	3.65	..	0.33
Fumaric	0.50	0.78	0.72
Succinic	1.02	0.64	0.65
Glycollic	2.06	0.47	0.51
Malic	2.40	0.43	0.45
Isocitric	2.75	0.40	0.41
Glyceric	2.95	0.38	0.38
Tartaric	3.20	0.36	0.32
Glucose	5.75	0.24	0.20

* Taken from Bassham (7); cf. also Benson *et al.* (10).

Additional evidence in favor of this view was obtained by Bassham (7, 10), who measured distribution coefficients of several radioactive organic acids, glucose and alanine in a butanol-propionic acid-water system. R_f values were determined from chromatograms developed in the same solvent. Using the value determined for alanine as a standard, Bassham evaluated the unknown terms in the equation of Consden *et al.* (33, 94) relating R_f to distribution coefficient. Thus

$$R_f = \frac{A_L}{A_L + \alpha A_S} = \frac{\text{excursion of band}}{\text{excursion of mobile phase}}$$

where A_L = fraction of cross-sectional area occupied by mobile phase; A_S = fraction of cross-sectional area occupied by stationary water phase, and α = distribution coefficient = (conc. of solute in stationary phase)/(conc. of solute in mobile phase). Multiplying both numerator and denominator by $1/A_L$ gives

$$R_f = \frac{1}{1 + \alpha A_S/A_L}$$

The term A_S/A_L is constant for a given set of conditions (paper, solvent, temperature); α is a characteristic of the solute and determines the value of R_f, i.e., position on the paper.

For alanine, the measured value of α in the n-butanol-propionic acid-water system was 3.65; R_f as measured from the chromatograms was 0.33. Substitution into the equation permitted evaluation of the constant, $A_S/A_L = 0.55$. R_f values for other solutes in the same system may now be predicted from their distribution coefficients according to the equation

$$R_f = \frac{1}{1 + 0.55\alpha}$$

Table 1 affords a comparison of R_f values calculated from the equation by substituting independently determined values of α with those measured directly from the chromatograms. The data offer evidence in support of the conclusion (33) that the paper functions primarily as an inert support and that α is the principal parameter which determines the R_f value. Bate-Smith and Westall (9), from a study of the chromatographic behavior of phenolic substances and derivatives, similarly support this interpretation.

Not all published data agree, however, with an explanation of function based entirely on liquid-liquid partition. A number of deviations from predictions according to this theory were noted by Moore and Stein (see Table 1, p. 272, in (102)) working with amino acids on starch columns. Taking data from Consden *et al.* (33), they cross-compared R_f values on paper, band rates on starch and directly determined partition coefficients. Their results led them to question the applicability of the liquid-liquid theory to either starch or paper. Additional experience confirmed their doubts (135).

By a simple experiment Moore and Stein (Fig. 6 in (102)) effectively demonstrated that adsorption does function on a starch column. They achieved complete separation of alanine and glycine using only water as developing solvent. Likewise, leucine, phenylalanine and tryptophane were separated with either water or 0.1N HCl. With these simple solvents the band rates show considerable acceleration. This makes it difficult to assess the data for the degree of adsorption with the usual binary or ternary solvents.

Arden and co-workers (2), also using water miscible solvents for the separation of inorganic ions, agree that the liquid-liquid concept is inadequate for a complete explanation of the process on paper supports. Lugg and Overell (89) were obliged to conclude from their observations on organic acids that adsorption is considerable in the absence of suppressing agents. Finally, by showing that amino acids can be recovered from areas of the paper over which they passed during development, Jones (77) showed conclusively that adsorption does occur. Other workers who discussed the problem (12, 40, 64, 83) considered the process more involved than simple liquid-liquid partition. Martin (95a) has reviewed some aspects of the problem.

This treatment is not primarily concerned with the present highly controversial subject of the theory of paper chromatography. It would appear better to leave the question open and consider that the relative rates of movement of the spots are governed as postulated by Consden *et al.* (33) except that the partition ratio need not be restricted to that of a liquid-liquid system. What matters is the over-all partition ratio in the *presence* of the adsorbent or supporting agent. A solid support is always present; it may, or may not, play a role in determining the partition ratio. Similarly an aqueous phase, which alone would be immiscible in the mobile phase, may be present. Yet its effect in determining α and the R_f value could be secondary to that of adsorption. Solid-liquid and liquid-liquid partition ratios often depend on similar properties of the molecule. Consequently correlation of R_f values with liquid-liquid partition ratios cannot be entirely conclusive for ruling out adsorption effects.

The broader view is better from the practical standpoint since in choosing a system one is not restricted to water-immiscible solvents (11, 12, 64). In certain applications water-miscible solvents such as propanol and ethanol, or even water itself, may be superior.

APPLICATIONS

Whatever the principles involved, the utility of paper chromatography has been amply demonstrated by its wide applications to problems of qualitative analysis. Reports of applications to studies of intermediary metabolism (6, 31, 47, 57, 100, 107, 124, 130, 139, 164) or to in vitro enzymatic studies (14, 75, 132, 154) have not been as numerous as applications to amino acid, protein and peptide analysis. However, owing to inherent sensitivity and easy extension to tracer technique (3, 7, 10, 16, 25, 26, 52, 53, 137, 142, 145, 146, 148, 149), use of the method in metabolic studies is bound to increase.

Used with tracers, it shows great promise since the combination is capable of revealing intermediates at concentrations which by conventional means would have a low probability of detection. Because duration of exposure can be regulated in accordance with the intensity of radiation, x-ray film is both the most sensitive and the most convenient means of detecting spots of weak activity. Spots containing only a few counts above background of C^{14} radiation expose the film weakly but detectably in about 3 weeks. Finding these by scanning with counting devices would be inexpedient. After exposure, the processed film provides a key to the location of the radioactive compounds, which may then be counted in situ or excised and eluted for further study.

Paper chromatography may be used to provide evidence of the absence of a stable compound from biologic materials. Thus Consden *et al.* (38) disproved the report that nor-leucine occurs in spinal cord. Likewise, Joslyn and Stepka (78), by setting an upper limit for the concentration of dopa in extracts, adduced evidence against its participation in the browning of fruit tissues. Information of this sort may either save efforts to isolate a nonoccurring compound or indicate an alternate approach.

It should not be inferred from this that paper chromatography by itself is capable of detecting every component in any preparation. The quantity of any 1 compound on a chromatogram is governed by the amount of starting material that may be applied to the origin without overloading. A component may therefore be present below detectable limits unless fractionated and concentrated before application.

Already almost all the groups of biologically important compounds have been investigated by the paper technique. A partial list includes: adrenalines (72); alkaloids (105); anthocyanins (8, 9); inorganic ions (2, 23, 45, 118); flavines and their nucleotides (41); angiotonin (hypertensin) (49); keto acids (27, 82, 91); organic acids (10, 19, 20, 79, 89, 134); phosphate esters (10, 31, 64); penicillins (60, 157); porphyrins (126); purines, pyrimidines and nucleosides (50, 68, 70, 92, 93, 131, 151–153); steroids (24, 84, 85, 165, 166); streptomycin (69); sugars (55, 56, 65–67, 73, 74, 108–112). The list may be extended by consulting reviews (13, 30, 30a, 32, 39, 95, 95a, 139).

Application to the study of amino acids, either free or from protein and peptide hydrolysates, has been extensive (1, 5, 33, 37, 45–49, 63, 76–78, 94, 99, 116, 119, 120, 124, 128, 138–140, 144, 147,

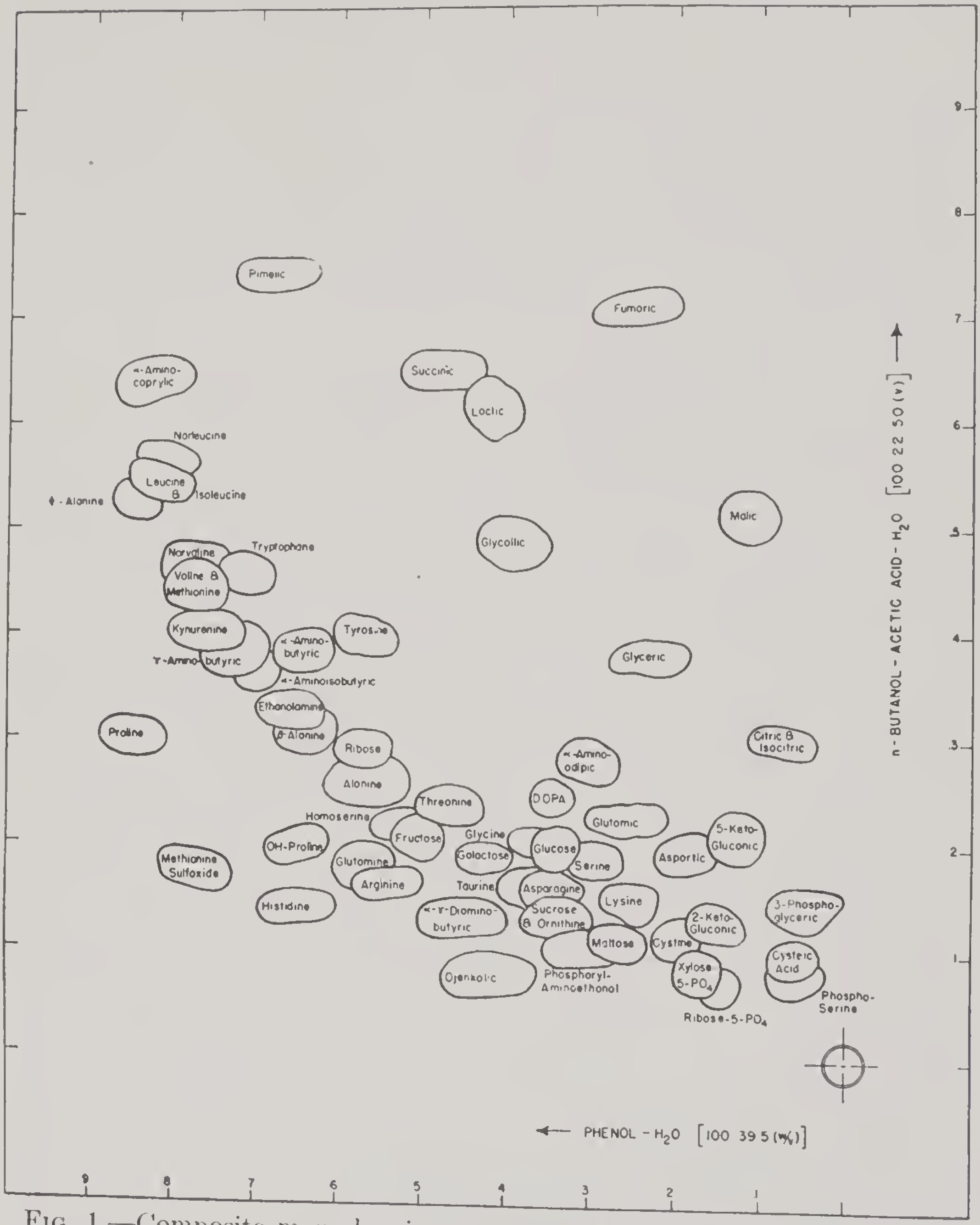

FIG. 1.—Composite map showing average relative positions of some commonly encountered compounds run on Whatman no. 1 paper ($22^1/_2 \times 18^1/_4$ in.) in the solvents indicated. Solvents were run to the edge, phenol first. Alanine and serine served as standards in each run. The author is indebted to Dr. F. H. Stodola and L. B. Lockwood for samples of the ketogluconic acids; to Dr. C. E. Dent and R. J. Brown for γ-aminobutyric acid; to Dr. R. Y. Stanier for kynurenine, and to the Levene Memorial Collection for the pentose phosphates.

154, 162, 164). For these the procedure generally follows the techniques outlined later. Determination of peptide structure requires additional steps. A scheme for identifying and determining the structure of peptides involves the following sequence: ionophoretic fractionation (34); resolution of the fractions on paper; elution of the individual peptide; hydrolysis of an aliquot of the eluate followed by chromatography to determine component amino acids. End-groups are determined by deaminating another aliquot of the eluted peptide with nitrous acid, hydrolyzing the deaminated peptide, and again chromatographing the hydrolytic products. Comparison of the 2 chromatograms identifies the terminal residues by their absence from the 2d chromatogram owing to the loss of their amino groups. Full details are given in the original papers (36, 37). Dent (45) has described a similar method for end-group determination. Sanger (128) used 1:2:4-fluorodinitrobenzene for the same purpose. Jones (77) and Sanger (in (13)) reviewed the subject of peptide analysis.

Choice of method.—Whether the usual descending (33) or the more recent ascending method is chosen depends in part on the nature of the problem and the equipment available. Williams and Kirby (155), who introduced the ascending method, claimed for it the advantage of yielding sharper spots. However, experience indicates that the descending method can produce spots of equal sharpness. Within limits, better definition is obtained with slower rates of solvent flow which the ascending method automatically provides. Still, whenever it is necessary to employ the full area of a standard filter paper sheet the slight improvement attributable to this factor scarcely justifies the extra time required for ascending chromatograms (for comparative rates see Table 1 in (155)). That adequate resolution of complex mixtures requires full utilization of large sheets can be inferred from Figure 1. Other variables besides rate of solvent flow contribute to spot shape (33, 46, 77, 104); hence on considerations of the time factor and possible complexity of the sample, the advantage is with the descending method.

However, because of its simplicity the ascending modification is becoming increasingly popular and for simpler mixtures has considerable utility. As originally described (155), full sheets were not used and the papers retained their shape even when wet. Other workers (73, 90, 163), using sheets of standard size, found it necessary to devise supports to prevent collapse. This can be achieved more simply by using the cabinet described later. A large tray on the bottom holds the solvent into which the sheets hang from wooden bars resting on the supporting cleats at the narrow sides. In this way a single cabinet can accommodate 20 sheets. Since cylinders do not have to be formed, handling is simplified and space conserved.

ASCENDING PAPER CHROMATOGRAPHY

APPARATUS AND SUPPLIES

a) *Cabinets.*—These should be vapor-tight to assure a saturated atmosphere with respect to the developing solvents (33). Unless cleanable, it is best to reserve a separate cabinet for each solvent. The cabinets described here are a modification of Dent's design (46). They are built from $^3/_4$ in. plywood to the following inside dimensions: 27 in. long, 18 in. wide and 26 in. deep. Cleats $1^1/_4 \times 1^1/_4$ in., attached to the inside of the 18 in. wall 3 in. below the rim, serve as rests for the trough bases (Fig. 2, *d*). A reinforcing wooden strip 1×2 in., flush with the rim around the outside of the cabinet, provides both rigidity and a firm anchor for the locks. End windows 12×20 in. permit observation of all papers during the run. The lid, also of $^3/_4$ in. plywood, is $30^1/_2$ in. long and $21^1/_2$ in. wide. The vapor seal is made from gum rubber tubing cemented in a rab-

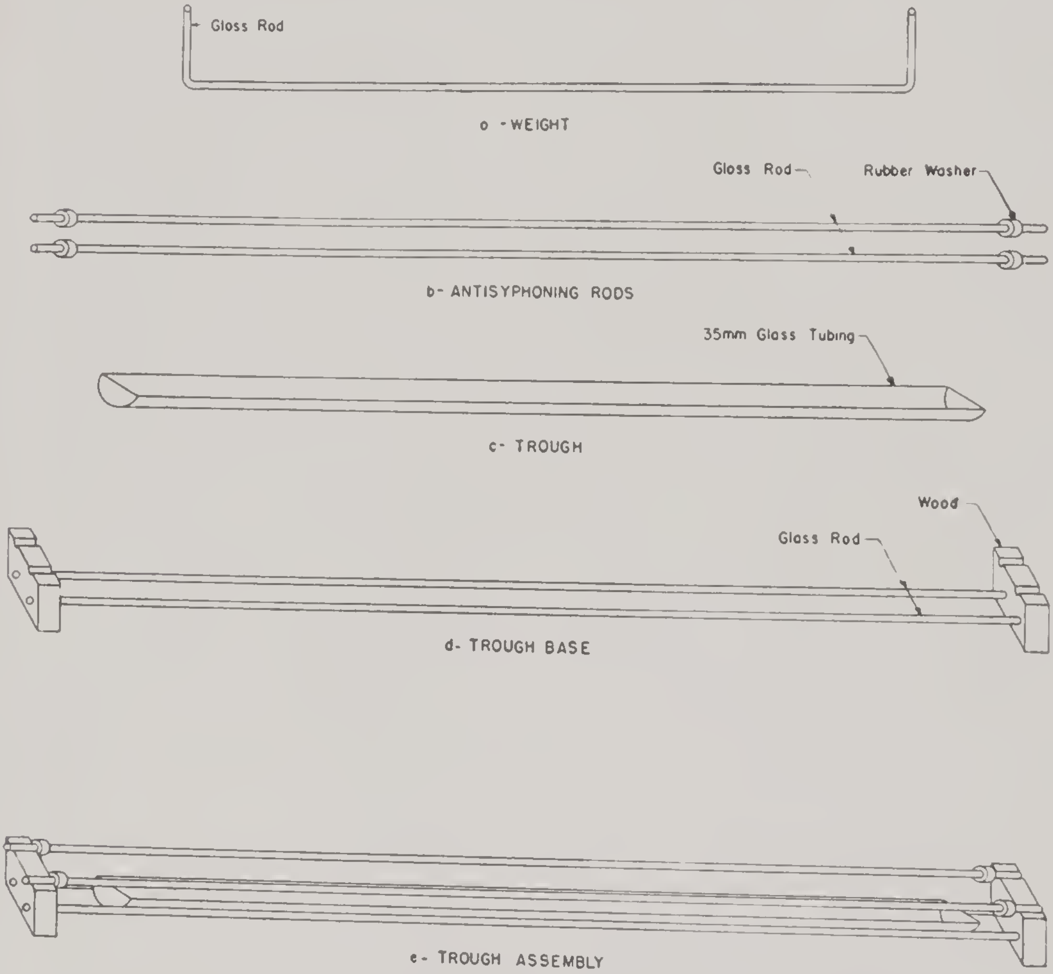
Glass Rod
a - WEIGHT
Glass Rod
Rubber Washer
b - ANTISYPHONING RODS
35mm Glass Tubing
c - TROUGH
Wood
Glass Rod
d - TROUGH BASE
e - TROUGH ASSEMBLY

FIG. 2.—*a–d*, components of trough assembly; *e*, components assembled. For description, see text.

beted groove along the rim. Trunk hasps or sash fasteners, 2 per side and 1 per end, secure the lid. Locks are attached to the reinforcing strip; the striker plates to the edges of the lid. Two asymmetrically placed guide pins in the reinforcing strip, matching corresponding holes in the lid, assure proper fit and orientation of the lid.

The inside of the cabinet can be heavily impregnated with paraffin; but Formica paneling, found to be resistant to most solvents used, makes a superior lining. Paraffin-lined cabinets may require "aging" in the presence of the solvent before fully reproducible chromatograms are obtained.

b) *Trough assembly* (Fig. 2, *e*).—The trough base *d* is easily constructed from blocks of wood and 26$^1/_2$ in. lengths of 6 mm glass rod. The notches in the end plates are spaced to hold the antisiphoning rods *b* about $^1/_8$ in. above the rims of the trough *c*. Papers hanging directly over the rim are prone to siphon. The antisiphoning rods are also made from 26$^1/_2$ in. lengths of 6 mm glass rod. Short pieces of rubber tubing near the ends prevent the rods from slipping out of their notches during manipulation. The weight *a* should be made from heavier rod or from 6 mm tubing filled with lead shot. This assembly, like the self-contained troughs of Steward *et al.* (141; see also, 162), permits loading outside the cabinet.

For troughs, glass is the one material consistently resistant to chemical action of the solvents. Although stainless steel has proved satisfactory for some workers (10,† 110, 162), an instance of excessive phenol oxidation was traced directly to a stainless steel trough (133). Whether a given type will be satisfactory for phenol solvents seems to depend on its composition; none are completely resistant to HCl. This point merits consideration in the choice of equipment if use of HCl is anticipated.

Glass troughs (Fig. 2, *c*) can be made by sawing through a length of 35 mm Pyrex tubing with a diamond saw. Turning up the ends in a flame and grinding the sharp rims completes the trough. As an alternative, lengths of tubing may be split by the hot wire method, or the method of Longenecker (88) may be used.

c) *Paper*.—Stock filter paper should be kept scrupulously clean and protected from ammonia vapors. Adsorbed ammonia, in the absence of catalyst poisons, accelerates the oxidation of phenol during the run. This is undesirable because the oxidation products, if excessive, interfere with even flow of the second solvent. It is best to handle the paper only by the edges.

Because the fibers of most papers possess a degree of preferred orientation which influences solvent flow, R_f and spot shape (22,

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† Polished stainless steel, Type no. 316.

77, 104), chromatograms for comparative purposes should all be run with the texture oriented in the same direction. In practice this is accomplished by observing a standard routine in removing sheets from the stock.

Sheets of Whatman no. 1 or no. 4, $18^1/_4 \times 22^1/_2$ in., are most commonly used. Other papers were considered to have advantages by some workers (22, 28). Kowkabany and Cassidy (83) examined 75 papers with reference to suitability for chromatography and made recommendations based on their tests. Jones (77) stated that on Whatman no. 7 amino acids form round spots. Judging from the experience of Müller and Clegg (104), one might expect that on this paper other compounds would show a similar improvement in spot shape.

The effects of metals in the paper were discussed by Consden *et al.* (33, 35), who attributed a dark phenol front and "bearded" amino acid spots to the presence of copper. Both disturbances were eliminated when catalyst poisons such as H_2S or HCN were admitted into the cabinet, or if cupron were added to the phenol solvent. Hanes and Isherwood (64) also experienced difficulty with impurities in the paper. Besides copper they implicated lead and other cations in whose presence phosphate esters either failed to move or streaked. Washing with dilute acids corrected the difficulty. Hanes and Isherwood (64) therefore described an apparatus for washing paper in bulk.

Whether preventive measures of this sort will be necessary can be determined with a few trial runs without them. For amino acids and sugars it will be found that most batches of Whatman no. 1 and of similar papers can be used as supplied.

d) Solvents.—Of the many solvents described, water-saturated phenol (33, 46), "collidine" (33, 46, 48) and n-butanol-acetic acid-water combinations (77, 109) have proved most versatile. No combination of solvent systems in use will separate completely all the amino acids or sugars on a single chromatogram. This is probably true for other groups of nearly equal complexity.

Aqueous phenol is particularly useful for the amino acids and sugars. The solution should be colorless or nearly so; a colored solvent may cause difficulty by depositing oxidation products on the paper. Phenol can easily be purified to meet this specification by simple distillation at atmospheric pressure from an all-glass apparatus. Collecting the distillate in tared containers simplifies subsequent preparation of the solvent. When needed, the solvent can be prepared quickly by adding 39.5 ml. of metal-free water for each 100 g of phenol and warming in a bath with occasional stirring until solution is complete. On cooling to the working temperature the solvent is ready for use. Prepared in these proportions the phenol is slightly undersaturated and no aqueous phase should separate at temperatures above 20 C.

Consden *et al.* (33) described water-saturated s-collidine (2:4:6-trimethylpyridine) as a satisfactory solvent for amino acids. However, Dent (46) with Stepka and Steward (48) were unable to prepare a satisfactory solvent for amino acids with refined s-collidine from American sources. They obtained good results, duplicating those with s-collidine from British sources, by mixing 1 vol of 2:4-lutidine with 1 vol of 2:4:6-trimethylpyridine and equilibrating the resulting solution with 2 vol of water (48). The top phase was used. Dent (46) refers to this mixture as "collidine."

With the ternary system an occasional run was lost as a result of phase separation due to temperature fluctuation. To circumvent this defect a binary system containing 2:4-lutidine† and water was devised (78). A satisfactory combination, closely reproducing the amino acid pattern obtained with "collidine" (46), was obtained by mixing equal volumes of 2:4-lutidine and water. It is undersaturated with respect to water at temperatures below 25 C. Reducing the proportion of water raises the separation temperature.

The butanol-acetic acid-water solvent of Partridge (110), effective for the separation of sugars, was tested for the amino acids (136). Although the R_f values were low, the results suggested that increase of the water content might improve the separations. Accordingly, combinations from several points along the critical line of a phase diagram for the components were tested with 26 amino acids. The best pattern was obtained with the following formula (136): n-butanol, 100 ml; glacial acetic acid, 22 ml; water, 50 ml. Only freshly prepared solvent should be used because the properties change as esterification proceeds. This, however, does not reach disturbing proportions with fresh solvent during the 15 hr required for the run.

The amino acid pattern obtained with this solvent (Fig. 1) is again similar to the pattern obtained on "collidine" chromatograms. A few shifts occur, notably threonine, methionine and tyrosine. Neither cystine nor dopa decomposes to the extent they do (46, 122) on chromatograms run in basic solvents. Cystine may occasionally show a "tail" indicative of decomposition, but dopa appears to be stable.

Comment by Werner Hausmann

I have found (64a) that all the common amino acids can be separated on a single map with the ascending technique by 2-dimensional chromatography in the following systems. The 1st system is made from 200 ml of 2-butanol and 80 ml of 3 per cent aqueous ammonia. The 2d is made from 150 ml of 2-butanol, 30 ml of 88 per cent formic acid and 20 ml of water. In the ammonium system, which must be run first, the solvent is permitted to climb for 48 hr, the paper then dried and the solvent permitted to climb for a second 48 hr. In the 2d direction the solvent is permitted to climb for 24 hr and then repeated for another 24 hr period. This procedure gave good separation of the pairs ornithine-lysine, serine-glycine, valine-methionine, leucine-isoleucine which were troublesome in other systems investigated.

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† Koppers Company, Inc., Pittsburgh.

Although certain of the organic acids chromatograph well on phenol/butanol-acetic acid chromatograms (Fig. 1), some tend to form elongated spots in the phenol direction. Also, their position is subject to greater variation in response to changes in conditions. Lugg and Overell (89) attributed this to the different properties of the ionic species which may exist. They found that these effects could be suppressed by incorporating relatively strong acids in the solvents. Thus with acetic acid as a component, the organic acids formed sharper spots; formic acid was even more effective. Lugg and Overell (89) discussed the factors involved in selecting a suitable solvent for the organic acids, gave formulas for preparing several solvent mixtures and tabulated the associated R_f values for a series of acids. Brown (20) separated some of the carboxylic acids as anions.

The lower fatty acids can also be separated on paper chromatograms (19). Applied as sodium or potassium salts, the anions can be separated in solvents containing ammonium ion. One effective solvent used by Brown (19) was prepared by mixing: ethanol, 1 vol; n-butanol, 4 vol; and 3N aqueous ammonia, 5 vol. For reproducible results a fresh mixture should be used. Kennedy and Barker (79) found that in their solvent (1 cc conc. NH_4OH in 100 cc 95 per cent ethanol), when applied as sodium salts, the anions ran too slowly. They therefore recommended conversion to the ammonium salt before application by treating first with an equimolar amount of ammonium sulfate and then with free ammonia. Chromatographically such mixtures behaved as ammonium salts with suitable R_f values. Acetate, propionate, butyrate and caproate were well separated.

Suitable solvents for separating the sugars and some sugar derivatives have been described by Partridge (109, 110, 112). Jermyn and Isherwood (74), interested in separating pairs of sugars, studied a wide range of combinations and developed several effective solvents of general applicability for sugar separations.

Solvents for separating alkaloids on paper have been developed and described by Munier and Macheboeuf (105). They also discussed the principles for selecting effective solvents for this class of compounds.

References to a variety of solvents for specific applications may be found in the reviews already cited (p. 30).

SPECIAL SOLVENTS.—Complete separations even on 2-way chromatograms cannot always be achieved. Solvents capable of resolving overlaps are therefore necessary. Most of these have been concerned with the amino acids, although the information compiled by Jermyn and Isherwood (74) is useful for the sugars.

Before an overlap can be resolved it must be located and then excised. Any compound which either fluoresces in or absorbs ultraviolet light can be located by this means. Amino acids can be made to fluoresce on paper by prior heating (117). Once located, the overlap can then be cut out and eluted according to the techniques described by either Dent (45), Martin (94) or Consden *et al.* (36). Rerunning the eluate in a solvent known to separate the compounds in question shows which are present.

Another resolving technique (136) which eliminates elution may be used. The cut-out is sandwiched to a paper strip between 2 microscope slides held together by paper clips and the strip developed in the usual manner. As long as good contact between cut-out and strip is maintained the resulting spots are sharp. For this it is sometimes necessary to trim the cut-out to the dimensions of the slide.

Overlaps of the leucines and methionine can be separated by running in a system prepared by mixing equal volumes of n-butanol and benzyl alcohol and saturating the solution with water (33) or in water-saturated n-butanol (46). Work (164) has found tertiary amyl alcohol in the presence of diethylamine capable of separating leucine, isoleucine and phenylalanine. When valine and methionine overlap they can be separated in the following solvent (136): n-butanol-benzyl alcohol (1:1 v/v), 34.5 ml; glacial acetic acid, 9.6 ml; water, 16 ml. McFarren (101) has described a list of buffered solvents for amino acids. From his tables of R_f values it is possible to choose a solvent capable of resolving any pair of amino acid overlaps.

These techniques are not limited to resolution of overlaps but may also be used for verifications of identity. Here the suspected known is added either to the eluate or directly to the cut-out in the "sandwich" method, and the chromatographic behavior of the 2 is observed in several solvent systems. Identical behavior is presumptive evidence that known and unknown are the same. However, to have significance the tests must be conducted in dissimilar solvents.

PREPARATION AND MANIPULATION

The ensuing discussion, directed primarily to 2-way chromatography, applies also to the 1-way or "strip" method. Although the latter may have limited usefulness for certain work, it is valuable for exploratory runs to establish optimal aliquots, and for cochromatography in verifications of identity as described in the preceding section.

The variety of biologic materials which might be studied allows only general remarks about preparation of the sample. Where permissible, it is expedient to concentrate the sample so that only small aliquots ($< 100\ \mu l$) need be applied to the origin. Schedules should be designed to keep the quantity of inorganic salts to a minimum. If this is not feasible and they interfere (33), the sample may be desalted by the procedure of Consden *et al.* (36). If only the neutral components are of interest, salts may be removed with exchange resins (110).

To prepare the chromatographic sheet the sample is applied within a spot of 1 cm radius at 1 corner of the paper. Attempts to confine the sample within too small an area may be equivalent to overloading and thus result in streaked spots. The origin should be close to the solvent source to gain the full advantage of solvent travel, but it should not come in contact with the surface of the antisiphoning rod. Contact with wet surfaces may cause streaking. Similarly, the secondary origins for the 2d run should hang free on the far side of the rods. For the apparatus described, the origin may be centered at a point 6 cm from the 1st edge and, allowing for "fanning out" during the first run, 7 cm from the 2d edge.

The sample is conveniently applied with a micropipet joined to a tuberculin syringe for easy control. Aliquots greater than about 20 μl are best applied in successive portions to keep the origin within bounds. Operation in a stream of warm air, as from a hair dryer, speeds preparation. When the sample has been applied, the sheet, by creasing ($^1/_2$ in.) along a proximal edge, is prepared for the trough.

Two sheets can be loaded into each trough. The loading operation may be performed conveniently if the trough is assembled on a flat surface as shown in Figure 2, *e*. The creased edges of the 2 sheets, 1 from each side and passed over the antisiphoning rod, are then inserted into the trough, where the creases are made to interlock. The weight *a*, placed along the interlocked creases, holds the sheets in position. The loaded trough is ready for transfer into the cabinet.

With all troughs in the cabinet, solvent is added and the cabinet closed. For 2 standard sheets 75–90 ml of solvent is sufficient for development to the edge. Adjustment of the solvent level allows some control over flow rate.

For routine 2-way chromatograms the author, like Dent (45, 46), prefers to use phenol as the 1st solvent. Although in theory the sequence in which solvents are used should make little difference, in practice reversing the order does appear to have an effect—usually for the worse. Possibly the explanation is to be found in the greater effectiveness of phenol in carrying interfering substances in the paper along with the front, thus removing them from their sphere of action.

Addition of reagents such as ammonia, cyanide or coal gas is not recommended for routine work. Omission of these addenda precludes the possibility of spurious spots arising from reaction between reagent and components of the experimental mixture (139). Their use is best reserved for special purposes.

Development is continued until the solvent front reaches the edge of the paper, or nearly so. For phenol, this takes 22–27 hr. For some purposes it may be desirable to run the front off the paper, e.g., to improve separation between poorly separated spots. To do this effectively, some means must be provided to prevent a liquid bead from accumulating along the bottom of the paper. Otherwise solvent "piles up" within the paper and spots move at reduced rates. Hanes and Isherwood (64) attached an absorbent pad of filter paper to the lower edge of the sheet; other workers (74, 111) serrate the edge, providing points from which the solvent could drip.

Sheets can be removed from the cabinet with least difficulty by making use of the antisiphoning rods. Clipping each sheet to its rod enables both to be removed simply by lifting on the rod, which can further serve as a support for the sheet during the drying process.

Thorough drying is essential before the sheets can be run in the 2d solvent. Specially designed dryers may be constructed, but a good fume hood will dry phenol papers in 6–8 hr. Apart from the possible effects of heat on the experimental compounds (58, 114), drying with heat between runs is not recommended because it gives rise to considerable discoloration from enhanced oxidation of phenol.

When the papers appear and feel dry, they may be run in the 2d solvent. For this they are turned through 90° and processed as before. Treatment of the papers following the final drying depends on the information desired but usually involves detecting the compounds on the chromatogram.

DETECTION

The methods for revealing compounds on a chromatogram fall into 3 categories. Compounds may be detected by their behavior in ultraviolet light (fluorescence or absorption), by radioactivity, if tagged with tracer, and by the color they form with certain reagents when these are sprayed on the sheet. All 3 methods can be used on the same chromatogram. Thus in a tracer experiment the chromatogram may first be exposed to x-ray film to give a radioautograph. Next, it may be scanned with ultraviolet light to locate fluorescing or absorbing areas. Finally, it can be sprayed with a reagent to form colored derivatives.

Amino acids, not normally fluorescent, can be detected by this means if the chromatogram has been previously heated (114, 117). This method, however, is considered less sensitive than the color developed with ninhydrin (77, 139). Owing to their strong absorption, ultraviolet light is useful for detecting purines, pyrimidines and their derivatives (68, 70, 92, 93, 151).

In certain instances it is useful to have a completely general method of detecting organic compounds. One such method, based on acid permanganate, was described by Procházka (123). With a 0.03–0.05N solution of potassium permanganate, acidified with sulfuric acid to a concentration of 0.06–0.3N, most of the organic compounds tested could be detected as white spots on a purple to brown background. Pacsu *et al.* (108) used alkaline permanganate for detecting sugars and some sugar derivatives. The iodine reagent of Brante (18) was capable of detecting a wide range of substances and may prove useful as a general reagent.

Ninhydrin, which forms blue to purple colors with the amino acids and yellow with imino acids, is considered the most sensitive reagent for this group. Pratt and Auclair (122) list sensitivity thresholds for the amino acids and some derivatives. The reagent, applied as a spray, reacts slowly at room temperature but rapidly at temperatures between 70 and 100 C. Bull *et al.* (22) found that the color intensity increased with time after removal from the heating oven—about 30 per cent in 24 hr—and that treatment with steam for 5 min brought the colors to their final intensity.

Contrary to statements in some texts (p. 185 in (129)), ninhydrin is not specific for the α-amino acids (46, 103). Dent (46) gave an extensive list of substances which react typically, including non-α-amino acids and primary amines. Peptides also react (36, 37, 46). The ninhydrin spray as originally prepared (33) contained 0.1 per cent of the reagent in butanol, but 95 per cent ethanol may be conveniently substituted (139).

For some purposes, such as identification, it is convenient to have more or less specific tests. A few for the amino acids follow.

Pauly's diazo reagent (115) gives an intense red color with histidine, which is more easily detected than the dusky ninhydrin color (46). Phenol, which also reacts, can be removed by washing with benzene or acetone. The reagent is prepared by mixing equal volumes of (1) *one* per cent aqueous sulfanilic acid in 1N HCl and (2) 0.69 per cent aqueous sodium nitrite. The histidine area is sprayed lightly with this solution and, when the paper no longer appears wet, again with 10 per cent aqueous sodium carbonate. Previous treatment with ninhydrin does not interfere, but tyrosine also reacts, hence if applied to strips the test should be well controlled with markers.

For detecting the sulfur-containing amino acids, Chargaff *et al.* (28) used an iodine-azide reagent. With it cysteine, cystine, homocystine and methionine are revealed as white spots against a brownish background. The first 3 react rapidly, methionine slowly. With a modified reagent (71), methionine also reacts rapidly. Contrast between background and spot is increased by including starch. The background color slowly disappears and the sheet may then be sprayed with ninhydrin for the other amino acids. To prepare the modified reagent, add 1 g of sodium azide and 10 ml of I-KI solution (0.64 per cent I_2, 3 per cent KI aqueous) to 100 ml of boiled 0.5 per cent soluble starch solution. The iodoplatinate reagent of Winegard *et al.* (156) is more general; it can also detect cystathionine, lanthionine and derivatives containing oxidized sulfur. Both reagents work best only after the papers have been well freed of solvents.

Arginine and some guanidine derivatives (p. 188 in (129)) may be detected on chromatograms (34) by the red color formed with the Sakaguchi reagent (127), but the test is less sensitive than the ninhydrin reaction (46). However, since combined arginine reacts, the reagent can be used to detect arginine-containing peptides. Horne and Pollard (69) detected streptomycin on strips by spraying first with 0.5N sodium hydroxide, immediately afterward with 0.25 per cent α-naphthol and again after 2 min with 5 per cent sodium hypochlorite.

Glycine gives a characteristic greenish color with o-phthalaldehyde which has been used to detect this amino acid (113). Aromatic solvents disturb the reaction, hence the test cannot be applied to papers run in phenol. It could be used for reruns of glycine eluates in nonaromatic solvents (113).

Only a few of the many reagents for sugars can be described here. References to others can be found in reviews (67, 112).

Ammoniacal silver nitrate, introduced by Partridge (109, 110) as a spray reagent for reducing sugars on paper, is a sensitive general reagent for reducing substances. Compounds related to the sugars like the ascorbic and uronic acids (110), reductone and hydroxytetronic acid (112) also react. Inositol (110) and sugar alcohols also form dark spots but do so only slowly. Interfering contaminants in some solvents (112) and reducing unknowns from the sample impair the utility of this reagent. Norris and Campbell (107) listed 15 common metabolites, all reacting with alkaline silver nitrate.

To detect reducing compounds, the reagent, prepared by mixing equal volumes of 0.1N silver nitrate solution and 5N aqueous ammonia, is sprayed on the chromatogram, which is then heated at 100 C for 5–10 min (109). Black or brown spots mark the positions of reducing compounds. Washing in dilute ammonia (p. 410 in (51)) or photographic hypo removes excess reagent and prevents darkening of the background on exposure to light.

Aniline yields colored derivatives with aldoses when heated in the presence of acid. Aldopentoses give shades of red, whereas aldohexoses tend to give brownish tones. Partridge adapted the reagent for use on paper by substituting oxalic (112), trichloracetic (110) and phthalic (111) acids for strong mineral acids which char the paper. The aniline hydrogen phthalate reagent (111) is prepared by adding 0.93 g of aniline and 1.66 g of phthalic acid to 100 ml of water-saturated butanol. After spraying, the papers are heated at 105 C for 5 min. Ketoses may react weakly but aldoses react strongly, 1 μg of the latter being detectable. In the presence of low concentrations of phosphoric acid, aniline reacts readily even with the ketoses and disaccharides (21). At low concentrations phosphoric acid did not attack the paper.

Naphthoresorcinol has been used to differentiate ketoses from aldoses (110). For this, fresh reagent is prepared by mixing equal volumes of: (*a*) 0.2 per cent naphthoresorcinol in ethanol, (*b*) 2 per cent aqueous solution of trichloracetic acid. Red colors develop with ketoses when sprayed with the reagent and heated in a dry oven at 110 C for 5–10 min; aldoses react weakly. By heating in a humidified atmosphere the range is extended to pentoses and uronic acids, which then form blue colors in 10–15 min (110). A modified reagent prepared by adding 0.1 vol of orthophosphoric acid (sp. gr. 1.85) to solution (*a*) shows no specificity and forms colored products with a variety of sugars (21).

Other phenolic reagents and aromatic amines can be adapted for detecting sugars on paper (67). Reducing sugars on papers sprayed first with aqueous picric acid solution and then with 10 per cent aqueous sodium carbonate appear as reddish-brown spots on a yellow background after heating at 110 C for a few minutes (136).

Organic acids have been detected with various indicators, but for general use Lugg and Overell (89) recommended bromcresol green and bromphenol blue. *Forty* mg of the dye was dissolved in 100 ml of 95 per cent ethanol and titrated with sodium hydroxide to pH 5.5 for BCG and pH 4.8 for BPB. Sprayed with this reagent, acidic substances appear as yellow areas on a green or blue background. For nonvolatile acids chromatographed as the anions, Brown (20) used chlorophenol red (0.04 per cent aqueous) adjusted to pH 7; bright yellow spots were formed on a mauve background. Anions of the fatty acids have also been detected on paper with indicators (19, 79).

Alkaline picrate is a sensitive reagent for many substances, particularly for those with keto groups. Some, such as pyruvic, α-ketoglutaric and levulinic acids, react immediately in the cold. The range is greatly extended if the sprayed chromatogram is heated. Vivid reddish spots appear against a yellow background. Koštiř and Rábek (82) reviewed the range and sensitivity of the reaction. They prepared the reagent just before use by mixing equal parts of 3 per cent sodium hydroxide solution and saturated picric acid solution. Keto acids have also been detected as fluorescent semicarbazones after spraying with semicarbazide reagent (91).

A method for the detection of acyl phosphates after conversion to hydroxamic acids has been described (132). The hydroxamic acids were detected and estimated by their colored derivatives with ferric iron. Other esters could conceivably be brought within the scope of this technique (p. 358 in (51)).

Bioautography, a method employing biologic means for detecting compounds on paper chromatograms, is especially useful in chromatographic studies of growth factors and antibiotics. The method is potentially quite selective, depending on availability and choice of the appropriate organism. The publications of Winsten and Eigen (158) and of Long and Williams (87) illustrate the use of this method and provide additional references. Giri and Prasad (59), applied the same principles to detect enzymes separated on paper chromatograms.

IDENTIFICATION

Casual inspection of a paper chromatogram may provide useful heuristic information, but since the chromatogram is most frequently used for qualitative analysis of complex mixtures, this purpose is achieved only when the compounds on the chromatogram have been correctly identified. Correct identification poses many problems. Simple measurement of R_f values *does not* suffice, not only because these are subject to variation from uncontrollable or unknown causes but also because superposition of spots from complex mixtures is a possibility not to be overlooked. Radioautographs from work on the CO_2 assimilation by green plants (26) show that still unidentified compounds overlap some of the known compounds plotted on the map of Figure 1.

These considerations do not vitiate the method; they do emphasize the need for corroborative tests. A combination of several tests will usually be necessary to accumulate enough supporting evidence for fiducial significance. Dent (46) and Steward and Thompson (139) discussed confirmatory techniques applicable to amino acids. In the following discussion a general approach will be adopted.

The pattern of spots obtained by spraying the paper with an appropriate reagent will, by comparison with a map of standards, furnish a basis for *tentative* identification. Differences in the colors formed (122) provide additional clues. Thus for amino acids sprayed with ninhydrin, the prolines appear as yellow spots; β-alanine and aspartic acid, especially after basic solvents or with modified reagent (162), as blue spots. With experience, other differentiating characteristics for particular compounds may be recognized. If a spot does not correspond to a known position, a guess to possible identity can be made on the basis of certain relationships between structure and chromatographic position. Steward and Thompson (139) discussed the principles for the amino acids and related compounds. Similar considerations apply to the sugar series (110).

From a tentative identification one may proceed to build up evidence by specific color tests and other means. Thus o-phthalaldehyde (113) may be used for glycine. Even if a reagent is not completely specific it may still be used in situations where the interfering substance(s) can be ruled out.

A provisionally identified spot may be cochromatographed with an authentic specimen of the known. Identical behavior in several solvent systems having different properties provides strong evidence that known and unknown are the same. This technique becomes quite sensitive if the compound being identified is radioactive. Then comparison of the radioautograph with the sprayed chromatogram provides visual evidence of similarity in such respects as shape of spot and distribution within the spot. Techniques for cochromatography have already been described (p. 38).

By running the chromatograms in an acidic or a basic atmosphere and noting the behavior of the tested compound it is possible to determine whether a compound is acidic, basic or neutral (46, 139). Under acidic conditions the R_f values of weak acids increase, those of weak bases decrease; contrary relationships obtain under basic conditions.

When the compound to be identified is an amino acid, especially from animal sources, it is necessary to show that the spot is stable to acid hydrolysis. The great variety of peptides makes it possible for every amino acid position to be covered by a peptide (46).

Hydrolysis, either chemical or enzymatic, can be applied as a confirmatory test of identity. An eluate of the tentatively identified compound can be hydrolyzed under the conditions required to split the corresponding known and the hydrolysate rechromatographed. Finding the expected products is then added evidence for the correctness of the original identification. Enzymatic methods appear promising in this application since with them a degree of specificity can be achieved.

D-amino acid oxidase sprayed on chromatograms of insect blood was used by Auclair (4) to demonstrate d-amino acids in the blood. The keto acids liberated by the oxidase were detected with 2,4-dinitrophenyl hydrazine. Jones (76, 77) and Synge (144) also used the enzyme to identify d-amino acids on paper chromatograms.

For radioactive compounds the hydrolytic method can be extended beyond mere observation of scission products. Rate curves can be determined—sometimes on material from only a single spot. Comparison with curves obtained from authentic material again provides a criterion of identity. Further, the eluate from a radioactive spot may be converted to a derivative and, with added known carrier, submitted to a series of purification procedures (81). Unchanged isotopic concentration provides another standard for judging identity.

Chemical derivatives of the eluate also can be compared chromatographically with like derivatives of an authentic specimen in tests of identity. Dent's procedure (46) of converting methionine to its sulfone and cystine to cysteic acid illustrates the method. By demonstrating chromatographic identity of the ureides prepared from eluates of an unknown in potato extracts and from γ-aminobutyric acid, Steward *et al.* (140) obtained supporting evidence for the identity of the 2 compounds. Hirst and Jones (67) cite unpublished work of Hough *et al.* in which the latter identified sugars by the chromatographic products found after epimerization.

Disappearance of a compound following some specific chemical treatment is still another way of obtaining confirmatory data. Using this procedure, Norris and Campbell (107) produced evidence for the intermediary role of 2-ketogluconic acid in glucose oxidation by *Pseudomonas aeruginosa.* Crumpler and Dent (42) used the principle to distinguish between α- and non-α-amino acids.

Physical methods such as x-ray diffraction (29, 43, 125), ultraviolet (70, 92) and infra-red (125) spectroscopy offer further means of testing identity. X-ray diffraction as applied to eluates from paper chromatograms is described by Christ *et al.* (29).

SUMMARY OF PROCEDURE

The operations involved in performing a qualitative analysis on paper sheets are summarized here in sequence. The summary is intended as a guide to a typical analysis, but the techniques may be modified to suit the requirements of the individual investigator.

I. Preparation of sample: For convenience in subsequent application to the origin the sample should be concentrated so that no more than 20–100 μl need be applied to reveal the desired compounds.

Extracts of tissues may be concentrated directly, but hydrolysates usually must also be freed from excess inorganic catalyst. If this is a volatile acid like HCl, the excess may be removed by repeated evaporation to dryness over solid NaOH in an evacuated desiccator. When $Ba(OH)_2$ has been used to mediate an alkaline hydrolysis, the barium may be removed as the carbonate or sulfate. If an enzyme has been used to catalyze the hydrolysis, it usually need not be removed from the hydrolysate before concentration unless the amount added is great enough to interfere with chromatographic separation. In the latter event the enzyme protein may be removed after heat coagulation or precipitation with organic solvents.

Preparations may be concentrated either by removal of solvent at reduced pressure and slightly elevated temperature or by lyophylization. The concentrates or residues should be made up to some definite volume with an appropriate solvent. All manipulations should be conducted with due regard for the stability or chemical reactivity of the compounds under investigation.

II. Determination of optimal aliquot: For this the sample may be applied in a graded series of parallel spots along 1 edge of the paper sheet and chromatographed unidimensionally. After color development with an appropriate spray reagent, the intensity and shape of the bands under each origin serve as a guide for the aliquot to be taken for 2-way chromatography.

III. 2-way chromatographic separation: 1) Optimal aliquot is applied to the origin in 1 corner of an 18 × 22 in. filter paper sheet. The application may be speeded by working in a stream of warm air directed over the origin. Duplicates should be prepared.

2) Sheets are loaded into the trough assembly, transferred to the cabinet and irrigated with the 1st solvent (phenol-water). The solvent travels to the bottom edge of the sheet in 22–27 hr.

3) The sheets are removed from the phenol cabinet and dried in a current of air. Time required for adequate drying depends on the rate of air flow, temperature, humidity, etc.; 6–12 hr is usually required. The phenol may also be removed by washing the sheets in ether (58). Caution!

4) The dry sheets are irrigated with the 2d solvent (n-butanol-acetic acid-water) in a direction at 90° to that of the 1st run (15–20 hr required).

5) The sheets are removed and again dried in a current of air for 4–6 hr.

IV. Detection: 1) Radioautography. If the sample contained radioactive material the dry chromatogram is placed in contact with x-ray film. Depending on the amount of radioactivity present and on the minimal activity per spot which it is desired to detect, exposure may last from a few hours to 3 weeks. Proper orientation of the processed film with respect to the chromatogram is facilitated by asymmetrical marking of the chromatogram with radioactive ink (10) at 2 points. The radioactive areas can then be accurately located on the chromatogram simply by superposing the ink marks with their image on the film.

Reagents sprayed onto the chromatogram may show correspondence between radioactive areas and specific compounds as revealed by their colored derivatives. Or radioactive areas may be excised, eluted and the eluates studied by chemical or enzymatic means.

It is possible to obtain quantitative data on the radioactive areas by direct radioassay in situ or, preferably, by measuring the radioactivity in the eluates after plating the latter according to standard radioassay procedures.

2) Ultraviolet detection. When viewed under the light of an ultraviolet lamp a chromatogram may show fluorescent spots or quenching areas. The former are seen as bright spots against a feebly fluorescent background, whereas the latter (e.g., purines and pyrimidines) appear as dark spots against the brighter background.

The amino acids do not normally fluoresce but can be induced to do so by heating the chromatogram at elevated temperatures (114, 117).

3) Colored derivatives. Duplicate chromatograms may each be sprayed with a group specific reagent; e.g., ninhydrin for amino acids, indicator sprays for acids, and phenolic reagents for sugars. Individual members of each series will, if present in sufficient

concentration, be revealed by the characteristic reaction.

V. Resolution of overlaps: The detection procedures may reveal instances of poorly resolved or superposed spots such as valine and methionine (Fig. 1). These may be resolved by rechromatographing of the excised overlap as described under Special Solvents (p. 38).

VI. Identification: Comparison of the position of the spots on the chromatogram with a map of standards provides a basis for tentative identification. Confirmatory evidence can be built up by a combination of tests such as cochromatography, specific reagents, etc., discussed under Identification (p. 44).

QUANTITATIVE CHROMATOGRAPHY

Although Dent (44) had previously used visual comparison of spot strength with standards for approximate quantitative estimates of amino acids, the first systematic attempts at quantitative applications appear to be those of Polson *et al.* (120), who used a "spot dilution technique" as well as a direct colorimetric method.

In the dilution method the hydrolysate was spotted along the paper in a series of dilutions. A similar series from a composite standard of known concentration was also prepared. Both were chromatographed simultaneously. After development of color with ninhydrin the amount of an amino acid in the hydrolysate was determined by finding a spot of equal intensity and area for the corresponding amino acid in the standard series. Later Polson (119) used the method for estimation of amino acids in bacterial hydrolysates. The method suffers from the need for a large number of chromatograms to bracket the concentration of a given amino acid. On 1-way chromatograms superposition poses a serious obstacle to reliable estimation.

Other attempts to adapt the ninhydrin reaction to quantitative estimation of amino acids from paper chromatograms have been described (5, 22, 86, 106). Final judgment on these must await wider experience. However, from considerations of the factors reported to influence reproducibility and color yields (58, 103, 147) it appears that some modifications may be required to make these methods more dependable. Gordon (61) has given a general account of the requirements for quantitative chromatography.

Fowden (58) described a quantitative ninhydrin method for amino acids on paper chromatograms based on a systematic study of interfering factors. The essential features are: avoidance of heat during drying, prompt removal of phenol by washing with ether, location of the spots by fluorescence before cutting out, and displacement of adsorbed ammonia with sodium hydroxide before development of color with a slightly modified Moore and Stein reagent (103). Concentrations were determined spectrophotometrically. Under the conditions employed, a linear relationship obtained between optical density and concentration over the range 1–10 μg of α-amino nitrogen.

Thompson *et al.* (147) also described a quantitative ninhydrin method wherein the color is first developed directly on the paper under controlled conditions and then extracted from the spots for colorimetric estimation. Their procedure, however, requires specialized equipment and appears to have no advantages over the less involved technique of Fowden (58) which at the moment appears to be the method of choice.

Woiwod (159, 160) investigated the applicability of the copper method (121) for quantitative estimation of amino acids separated on paper. For accurate results the comparative approach had to be adopted since the theoretical ratio for α-amino nitrogen to copper was not realized. The method, applicable to quantities of 1–25 μg of α-amino nitrogen, has been fully described and its limitations discussed (161). Martin and Mittelmann (96), after exploring and discarding other possibilities, also turned to the copper complexes and determined the copper polarographically. They indicated that further work was necessary for final assessment of the method.

Keston and co-workers (80, 81) described an isotopic method for quantitative assay of amino acids on paper chromatograms. The amino acids were converted to the p-iodophenylsulfonyl derivatives and chromatographed as such. Using I^{131}- or S^{35}-labeled reagent, Keston *et al.* (81) were able to locate and count the derivative on the paper strip. A simple calculation, counts in resolved band divided by counts per mole of isotopic reagent, then gave the equivalents of amino acid. The method is general and can be applied to any compound for which suitable isotopic reagents are available, provided the derivatives are separable into discrete bands or spots. Here the greater resolving power of the 2-way chromatogram has obvious advantages over the 1-way strip.

Quantitative applications to paper-resolved sugars do not seem to have encountered the difficulties experienced with the amino acids. In the sugar field, progress has been more rapid, partly because reliable micromethods already existed and partly because the smaller number of sugars normally encountered in any one preparation permits 1-way development. In these circumstances 1-way operation is an advantage since larger quantities of material can be obtained from the individual bands.

Flood *et al.* (55) and Hawthorne (65) were the first to describe quantitative procedures for the sugars. Flood and co-workers applied parallel spots of the sugar solution along a paper sheet. After development, a reference strip was removed and sprayed to locate the bands. With this as a guide, areas for each component were marked out and excised from the main body of the chromatogram. Each cut-out and a paper blank were separately extracted with a small volume of water in a modified Soxhlet extractor. The sugar in the extract was estimated either with Somogyi's copper reagent or by colorimetric measurement of the formaldehyde obtained by periodate oxidation. For consistent results a correction based on performance of the reagent with a standard sugar had to be applied.

Detailed accounts of improved reagents and procedures were given by Flood *et al.* (56) and by Hirst and Jones (66). The latter based their method on the estimation of formic acid formed in the reaction between polyglycols containing vicinal hydroxyls and periodate. Under the conditions adopted, the yield of formic acid was about 96 per cent of the theoretical. After destruction of excess periodate with ethylene glycol, the formic acid was titrated with standard alkali and the concentration of sugar calculated from the data. The determinations are reported to be accurate within 2 per cent.

Hawthorne (65) used similar procedures except for the reagent. In this method the eluted sugars, redissolved in bicarbonate buffer (pH 10.6), were oxidized with hypoiodite. Excess iodine was then titrated with standard thiosulfate. *Forty* μg of sugar could be estimated with an error of about 5 per cent. In this procedure precautions must be taken against the loss of iodine through volatilization.

The purines, pyrimidines and nucleosides, owing to their characteristic absorption in the ultraviolet, can be assayed after elution by conventional spectrophotometric techniques. With these substances, as Hotchkiss observed (70), artefacts brought about in the preparation may assume some importance. For the details of preparative and assay procedures, the original accounts (70, 131, 152, 153) should be consulted.

Methods for quantitative estimation of keto acids (27, 91) and of acyl phosphates (132) have also been described.

In an attempt to correlate some characteristic of the spots with solute content, Fisher *et al.* (54) found, for undistorted spots of amino acids and sugars, a linear relationship between length of spot as measured in the direction of solvent flow and logarithm of solute content. They also found a correspondence between spot area and logarithm of concentration. These correlations were established with solutions of pure compounds; it is therefore unlikely that the methods will find easy extension to chromatograms of bio-

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logic preparations. Spots from these are rarely uninfluenced by materials from the sample and by variations in conditions.

The obstacles to absolute quantitative work are numerous, and although a few have been recognized and controlled, it is unlikely that all have yet been brought to account. Different workers have emphasized different difficulties. Fowden (58) found that for amino acids prolonged contact with phenol, especially at elevated temperatures, results in serious losses. He was unable, in contrast to Woiwod (161) and Jones (77), to show low recoveries presumably due to adsorption along the path. On the other hand, Woiwod (161) did not attribute any serious losses to reaction with phenol even though he dried his papers for 3 hr at 80 C. Thompson *et al.* (147) do not state the conditions under which their papers were dried, nor how long the amino acids remained in contact with phenol. Presumably, therefore, they do not attach great significance to these factors, although they do stress others such as temperature, humidity, oxidation (see also (103)) and concentration of ninhydrin.

Considering the picture as a whole, it would appear best at present—especially for amino acids—to adopt a conservative view and to regard the differences in emphasis as reminders of the considerable problems involved in obtaining truly quantitative results from paper chromatograms. This does not imply that the methods at hand are without utility or that attempts to improve on them are to be discouraged. The point has been stressed to stimulate improvements and to encourage the use of supplementary quantitative techniques until experience indicates otherwise.

REVERSE PHASE CHROMATOGRAPHY

Conventional paper chromatography is unsuited for separating substances having low solubility in water. Such substances travel with the front of the organic phase without separating. To overcome this difficulty, fast-moving compounds have been converted to derivatives with improved water solubility (165). Since this approach has limited application, a number of workers have attempted to devise a system of general utility for water-insoluble compounds. These efforts led to the development of the reverse phase chromatogram, in which a stationary nonpolar phase is substituted for the usual aqueous phase.

Boscott (17) first applied the principle, using a column of cellulose acetate impregnated with butanol. Shortly thereafter, Boldingh (15), working with esters of long-chain fatty acids, extended the concept to paper. Strips treated with latex served as the support for separations with nonaqueous solvents. For corticosteroids

Zaffaroni and co-workers (24, 166) satisfactorily replaced the water phase by such relatively polar organic solvents as formamide or propylene glycol. They used benzene or toluene as the mobile phase. This system, however, was incapable of resolving cholesterol from cholestenone (84). With these, satisfactory separations were obtained on Quilon§-treated paper, using methanol, ethanol or 80 per cent ethanol as solvents. Here the stationary phase was considered as consisting of the nonpolar ends of the stearic acid residues.

A similar approach was adopted by Kritchevsky and Tiselius (85), who used paper coated with silicone. On treated strips they were able to separate 6 steroids, including androsterone, isoandrosterone and testosterone. The strips were prepared by immersion in a 5 per cent solution of Dow Corning Silicone no. 1107 in cyclohexane, blotting off the excess and drying for 1 hr at 110 C. Following application of the samples, the strips were permitted to adsorb the stationary phase (chloroform). For this, they were kept 1 hr in an air-tight container saturated with vapors of the heavier phase separating from the following mixture: 6 vol water, 10 vol abs. ethanol, 10 vol reagent grade chloroform. The chromatogram was then developed, using as solvent the lighter, more polar phase from the same mixture.

CONCLUSION

The technique described here has been shown by its wide application to constitute a powerful analytical tool. It is available for even wider use in problems of physiology and biochemistry, especially in conjunction with tracer techniques. But, like any method, paper chromatography is subject to misuse. The chromatogram provides separation, not identification. Identification is a function of the investigator, who should take pains when dealing with new compounds to avoid being misled by wishful thinking. Inspiration is a valuable quality and, although it may ultimately lead to the correct answer, by itself it does not suffice to identify a compound.

Each investigator should fully appreciate the shortcomings of the method before attaching a particular label to a spot and should try to minimize any possible misconception by every available cross-check, including other fractionation techniques. The resolution of complex biologic mixtures is a formidable problem and should not be underestimated.

The biologic worker could easily be led to the view that paper chromatography largely replaces the need for identification by the methods of classic organic chemistry. It does for some purposes. On the other hand, it may be well to state emphatically that when the identity of a substance must be unequivocal there is no substitute for actual isolation of a sufficient quantity of the pure substance for ultimate analysis, study of physical properties, etc. Paper chromatography is one of the most useful supplementary tools for attaining this final objective.

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§ Stearato chromic chloride, E. I. du Pont de Nemours & Co., Inc.

Such a conservative view is based on the realization that many new compounds still exist in nature whose behavior on paper is as yet a matter of conjecture. Artefacts are often encountered even with well known compounds. The full power of the method can be realized only when cognizance is taken of its limitations as well as of its potentialities.

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ELECTROPHORESIS

LEWIS G. LONGSWORTH, *Rockefeller Institute for Medical Research*

Electrophoresis is the movement of charged particles in solution under the influence of an electric field. The most widely used method for study of this phenomenon is that of moving boundaries. In this method the particles, or ions, are not observed directly but their presence is inferred from the contribution that they make to some property of the solution such as the refractive index. Thus if a conducting solution from which the particles are absent is superimposed in a vertical channel upon the more dense one in which they are present, at the junction or boundary between the 2 solutions the refractive index changes from its value in the 1 solution to that in the other. With the aid of optical methods to be described, this change of refractivity with height can be observed and photographed and the boundary thus located. If, now, an electric current is passed through the solutions in the channel the particles will move, and the resulting shift in the position of the boundary is a measure of this movement. In practice, the channel is U-shaped, with the solution under observation occupying the lower portion, so that if the boundary in 1 side rises on passage of a current, that in the other side descends.

The velocity of a particle when the electric field acting on it is 1 v/cm is called its mobility, u. In a given medium the mobility is a characteristic property of the ion species or particle but generally varies with the nature of the solution. This is especially true of protein ions where the mobility depends upon both the pH and the ionic strength of the solution in which they are dissolved. To obtain definite mobilities, therefore, the initial boundary is usually formed between the 2 solutions that result from the dialysis of the protein solution against a relatively large volume of an appropriate buffer solution. The dialyzable buffer ions are then present on both sides of the initial boundary at concentrations corresponding to the Donnan equilibrium (10) and provide a conducting medium in which the protein ions can move, on passage of the current, without appreciable changes of pH or electric field strength at the boundaries.

The moving boundary method is not restricted, however, to mobility measurements. In the case of proteins and related materials it may be used for analysis of mixtures such as blood serum.

This application is possible because the mobilities of the various proteins that may be present in a mixture frequently differ from each other. Initially each species occupies the same portion of the channel, but on passage of the current the zone in which a given protein is present moves at a rate proportional to its mobility and thus progressively separates from zones occupied by species having different mobilities. Thus, in the ideal case, shown diagrammatically in Figure 18, the initial boundary in each side of the channel splits, on passage of the current, into a number of separate boundaries equal to the number of species having different mobilities. Below each of these separated boundaries a species is present at the same concentration as in the original mixture but absent above and is said to disappear across the boundary. Moreover, owing to the close proportionality between refractive index and concentration, measurement of the difference in this index across each boundary affords an electrophoretic analysis of the mixture.

As will be shown later, other properties of proteins can also be determined with the aid of the method, and it can be used for preparative purposes. From the foregoing outline of the moving boundary method it is clear, however, that the apparatus and experimental procedure must provide (*a*) for the initial formation of a sharp boundary between the protein and buffer solutions, (*b*) for the passage of a known quantity of electricity with a minimum of disturbance from the heating effect of the current, electro-osmosis and the electrode processes whereby the current enters and leaves the solution, and (*c*) for observation and photography of the boundaries. The manner in which these requirements are met will be evident from the description, in the following section I, of a typical experiment with blood serum in a 0.1M sodium diethylbarbiturate buffer of pH 8.6. In this and succeeding sections the experimental procedures presented in some detail are ones that have been developed, for the most part, in this laboratory. It is well to remind the reader, however, that the fundamental ideas underlying the adaptation of the moving boundary method for the study of proteins are due largely to one individual, Arne Tiselius (52), and that the method is justly known as the Tiselius method.

I. Electrophoresis in the Tiselius Cell

Assembling the cell.—The Tiselius cell is shown in perspective in Fig. 1, *a*. With the exception of the tubes of section *I* that connect with the electrode vessels (described on p. 97), it is constructed of glass plates cemented together with a chemically resistant flux and consists of 3 sections that may be slid over each other along the planes AA' and BB'. With these interfaces lubricated with a mixture of 2 parts petroleum jelly and 1 part mineral oil and the sections aligned as shown in the figure, a continuous, leak-proof, U-shaped channel, C–C', of rectangular cross-section runs through the cell. Figure 1, *b*, is a top view of the center section. As will be shown later (p. 93), the width, 3 mm, of the channel is the critical dimension in controlling convective disturbances, the depth, 25 mm, largely determines the sensitivity of the optical method since this dimension is parallel to the light path, whereas the resolving power for particles with similar mobilities depends mainly on the clear height, 86 mm.

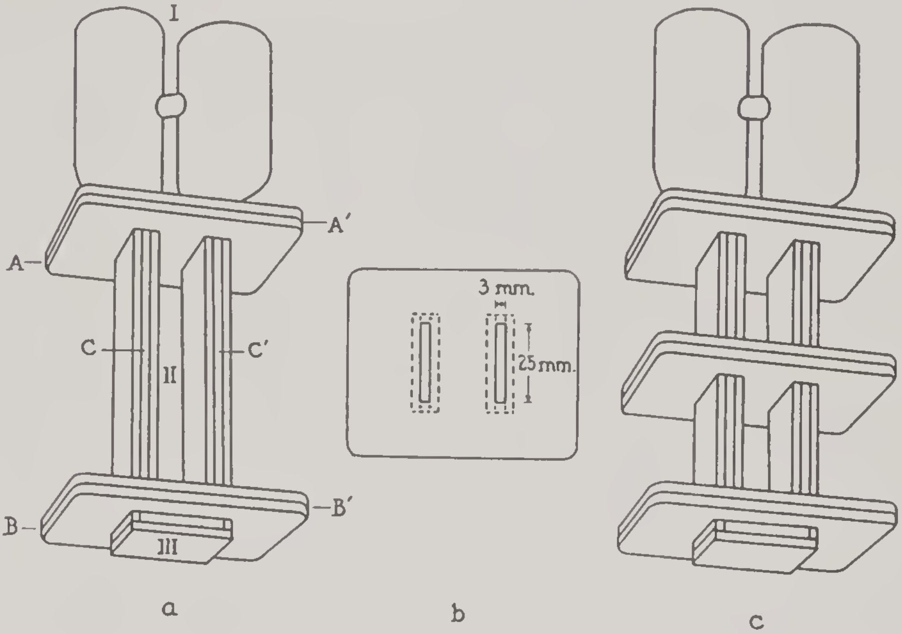
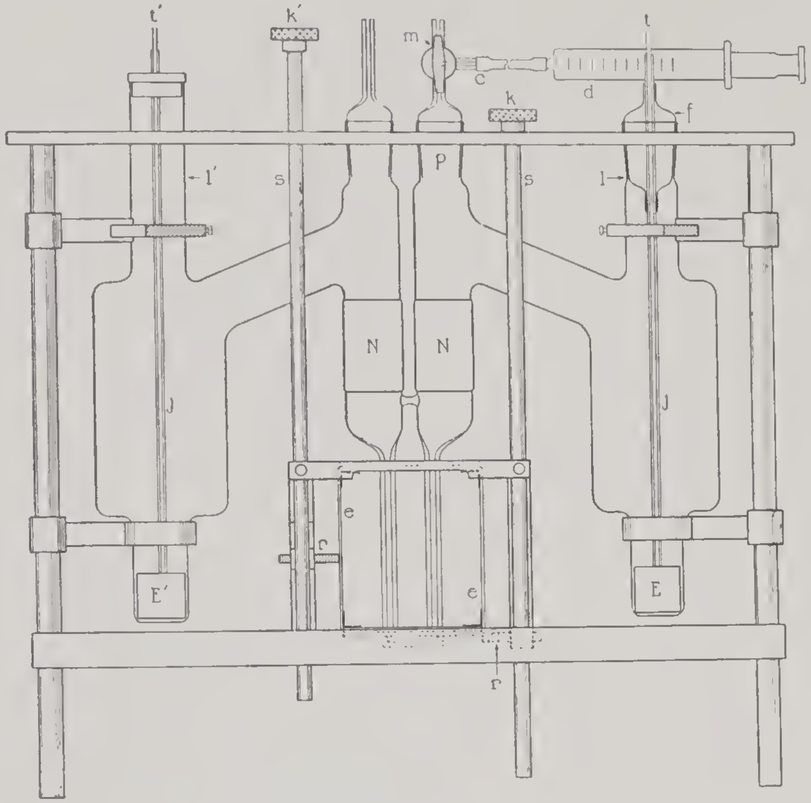


FIG. 1.—The Tiselius electrophoresis cell.

Filling the cell.—Following insertion of the metal clips *e* of Figure 2 between the horizontal plates of the center section, the assembled cell is placed in the support shown in the figure and the bottom section *III* filled with the protein solution, the filling extending slightly above B–B' (24). This is done with the aid of a 20 ml syringe and a 25 cm 17 gauge stainless steel needle. The sharpened tip of this needle may be used to puncture the dialysis bag for removal of the 12–15 ml of protein solution required for the experiment.

The contents of the bottom section are then isolated by displacing it to the left as shown diagrammatically in Figure 3, *a*. The displacement is effected with the aid of the knob *k* of Figure 2, whose shaft carries a pinion engaging the rack *r* that presses against the edge of the bottom section. Moreover, since the shaft *s* can be


t′
k′
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c
d
t
f
k
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l′
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l
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E
r

FIG. 2.—Electrophoresis cell, electrode vessels and support.

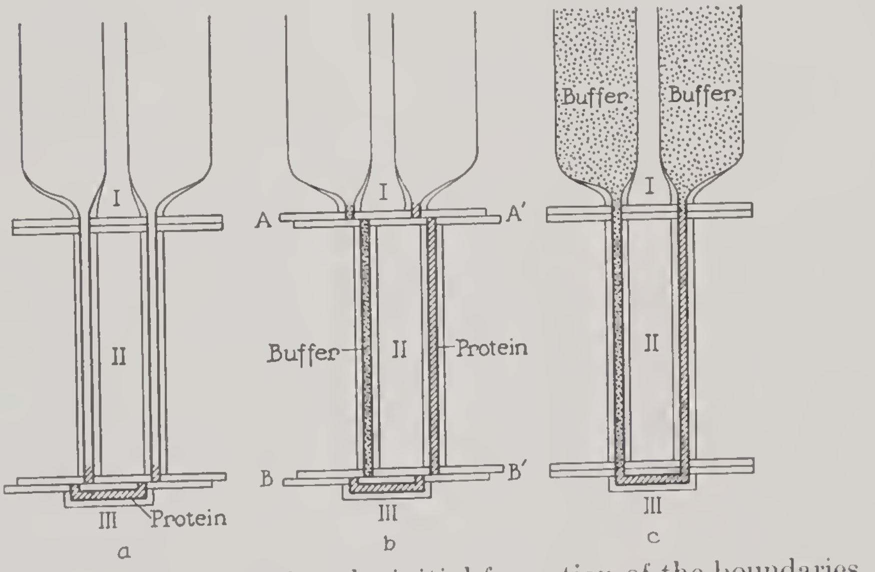
I
II
III
Protein
a
A
A′
I
Buffer
II
Protein
B
B′
III
b
Buffer
Buffer
I
II
III
c

FIG. 3.—Diagrams illustrating the initial formation of the boundaries.

raised, the rack can also be brought to bear on the insert *e* if it is necessary to shift the center section to the left. A similar device on the left permits shifting either of these sections to the right.

The right side, say, of section *II* is now filled with the protein solution and the left side rinsed and filled with buffer, the levels in both sides being above *AA′*. With the aid of neoprene sleeves, *N* of

Figure 2, the cell is connected to the electrode vessels and these are filled with buffer to a level just below their side-arms. This connection is less likely to leak if the glass surfaces making contact with the sleeves are lightly greased with petroleum jelly. Moreover, 1 end of each sleeve is provided with a neoprene ring on which the remainder of the sleeve can be rolled. With a sleeve thus rolled up it is slipped over the lower end of the side-arm, which is next swung into position above the top section of the cell. The sleeve is then allowed to unroll into the position shown in the figure, thus making the desired connection.

The assembly is now placed in a thermostat regulating at 0.5 C, a value whose significance is considered on page 95. After allowing a few minutes for thermal equilibration the center section is displaced to the right and the bottom section returned to its original position (Figure 3, *b*). The excess protein solution is then rinsed out of the top section and both sides of this section, together with the attached electrode vessels, filled with buffer to the level *ll′* of Figure 2. The silver-silver chloride electrodes *E* and *E′* are next inserted, care being taken not to trap air bubbles as the ground-glass stopper *f* is seated. To provide chloride ions for the electrode process 25 ml of 1M sodium chloride is allowed to flow slowly down each of the glass-enclosed silver tubes *t* and *t′*. As the buffer around the electrode *E*, for example, is displaced, the liquid rises into the hollow stopper *p* and the excess overflows through *c*. With the aid of a rubber cap the tube *t* is closed while still filled with solution and the capillary rubber tube *c*, also filled with liquid, is connected, as shown in Figure 2, to the syringe *d*, this having been previously filled with buffer and clamped in the "compensator." This device, not shown in the figure, imparts, with the aid of a small synchronous motor, a uniform movement at the desired rate to the piston of the syringe. By turning the stopcock *m* through 90° from the position shown in Figure 2, the right-hand side of the apparatus is closed, connection with the compensator being retained. It will be noted that air is excluded completely from this side, whereas that on the left remains open to the atmosphere.

Forming the boundaries.—Now, by returning section *II* to the center position (Figure 3, *c*), sharp boundaries between the protein and buffer solutions are formed at the junction of *I* and *II* on the right and between *II* and *III* on the left. Before current is passed they are shifted into view from behind the opaque horizontal plates of the cell by means of the compensator. Injection of buffer from the syringe at the rate of 3.6 ml/hr for 25 min will shift them to the level indicated by the schlieren band at H_i in Figure 4, *c*. Before the boundaries have emerged, however, the "base lines" for the patterns are recorded, that for 1 side of the channel being shown at *b* in Figure 4. This procedure is described under Schlieren methods (p. 71).

Testing for leakage.—It is also convenient to test for leaks during the compensation. This is done by connecting 1 lead from the power supply to an electrode and the other to the cell frame, i.e., the bath water. On application of a potential difference of 100–200 v a current of as much as a few microamperes usually indicates an improperly assembled cell with the possibility of loss of solution during the experiment.

After shifting the boundary the compensator is stopped and the syringe isolated by turning *m* of Figure 2 through 45°. If not shifted too rapidly the slight spreading of the boundaries that occurs during this process is due mainly to diffusion and for most purposes can be neglected. This is not true, however, if a boundary is formed initially in a stopcock as in some cells. In this case a siphoning technique, such as the one suggested by Kahn and Polson (19), can be used to resharpen the distorted boundary, after it has been shifted into the channel but before current is passed.

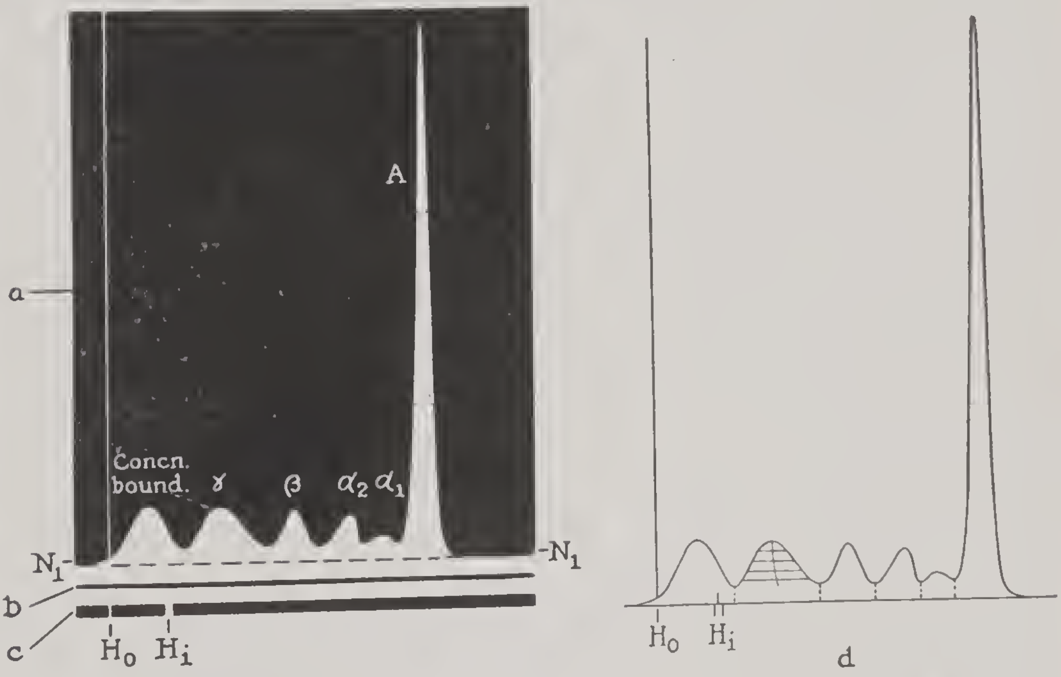

FIG. 4.—Negative and tracing of the pattern of the rising boundaries of a human serum.

Starting electrophoresis.—Following the recording of the initial boundary positions, e.g., Figure 4, *c*, a potential difference of 135 v, say, is applied across the terminals *t* and *t′* of Figure 2. At pH 8.6 the serum proteins are present as anions and the electrode connecting the left-hand side of the channel in Figure 2 must be positive so that the boundary will rise in this side and descend on the right. The value of the current i is determined potentiometrically from the potential drop E across a known resistance R, connected in series with the moving boundary cell, i.e., $i = E/R$. If the potential of the power supply is reasonably steady the current drift during an experiment amounts to no more than 1 or 2 per cent. The average of several readings of the current, when multiplied by the time, affords a value for the number of coulombs passed that is considerably more accurate than this. If precise values of the mobilities are not required, the current can be measured with the aid of a milliammeter.

Once the electrophoresis is started the investigator is largely free to measure the pH and conductance of aliquots of the protein and buffer solutions (pp. 87 and 90) to determine mobilities and concentrations from previously recorded patterns (p. 80) and to prepare solutions for subsequent experiments (p. 87).

After the boundaries have separated sufficiently to give well defined minima in the gradient curves, the current is interrupted and the pattern of both sides of the channel recorded. Figure 4, *a*, is the negative of that side in which the boundaries ascended. If none of the separated fractions are to be recovered as described under Preparative and Countercurrent Electrophoresis (p. 98), this concludes the experiment.

Patterns can also be recorded while the current is flowing. In mobility determinations, however, the 2 patterns from which the displacement of a boundary is evaluated should be obtained either with the current flowing or with it interrupted. The temperature rise accompanying the passage of the current results in small volume changes.

II. Schlieren Methods

Optical principles.—Although optical methods based on the interference of light are being adapted for work with moving boundaries, and are considered in the next section (p. 76), the Foucault-Toepler schlieren effect introduced by Tiselius (52) is now widely used. The form that the schlieren method takes for the observation and photography of the refractive index changes at the boundaries is shown diagrammatically in Figure 5. Here a spherical lens L, called the schlieren lens, forms at Y_0 an image of the illuminated, horizontal slit S, called the source slit. The camera lens O is focused on the channel C and forms an image of it on the screen at G. If the fluid in the channel is homogeneous this image will be uniformly illuminated. Suppose, however, that a protein-buffer boundary is present. Such a boundary consists of a region B, of finite thickness, in which the refractive index n varies continuously with the height h from its constant value in the body of the protein solution to that in the buffer. The gradient, $\mathrm{d}n/\mathrm{d}h$, of refractive index thus varies from zero to a maximum and back to zero.

In Figure 5 the variation of the gradient in the boundary is indicated by the density of the shading. Each layer of solution in the boundary acts like a prism and deflects the light passing through it. With but few exceptions, e.g., aqueous ammonia, the denser solution below the boundary has the higher refractive index and the deflection is downward. For a sufficiently thin layer the gradient may be considered as constant and the light traversing this layer forms an image of the slit below the normal one at Y_0. In Figure 5, 2 such displaced images are indicated. That at Y_m is formed by the layer in the boundary at h_m having the maximum value of $\mathrm{d}n/\mathrm{d}h$. That at Y_1 is the superposition of the 2 images formed by the 2 layers at h_i and h_j for which $\mathrm{d}n/\mathrm{d}h$ has identical values. Actually the displaced slit images formed by all the layers in the boundary combine to give a rectangular pattern of interference fringes, the so-called Gouy fringes, in the region from Y_0 to Y_m (27).

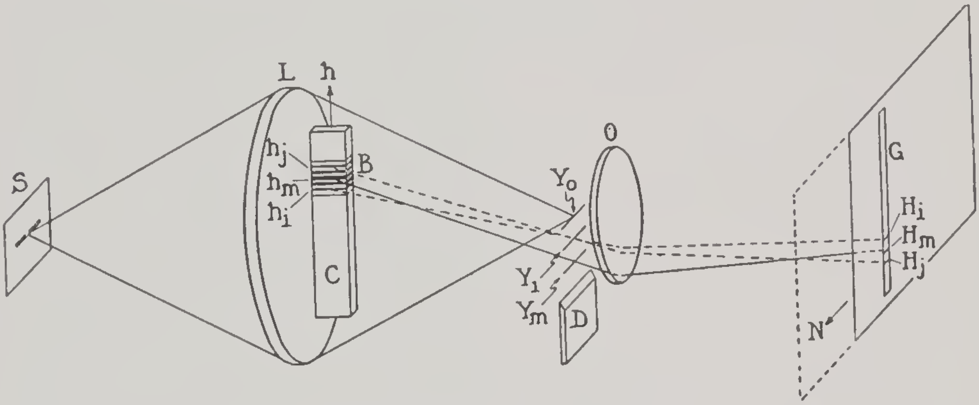

FIG. 5.—Schlieren method for study of refractive index gradients between solutions.

If, now, an opaque screen D, called the schlieren diaphragm and having a sharp, horizontal upper edge, is placed in the plane of the slit images at Y and raised to the level Y_m, the light through the layer at h_m in the boundary will be intercepted and a dark, narrow band will appear at the conjugate level H_m in the image of the channel. On raising the diaphragm farther, e.g., to Y_1, the light from h_i and h_j, together with that from all intervening layers in the boundary, will be intercepted and the dark band in the image of the channel will broaden until the edges of the band are at the conjugate levels H_i and H_j. This, obviously, can be continued until the undeflected rays at Y_0 have been intercepted and the entire image of the channel becomes dark.

The scanning process.—With a single boundary in the channel the series of 14 photographs shown in Figure 6, *a*, was taken with the schlieren diaphragm raised a fixed distance between each exposure. The progressive broadening of the schlieren band is apparent. In the schlieren scanning procedure (22) the stepwise recording shown in Figure 6, *a*, is made continuous. The central portion of the life-size image of the channel at *G*, Figure 5, is masked by a vertical slit 0.2 mm wide and the photographic plate moved horizontally in the direction *N* at a constant rate across this slit. Actuated by the same mechanism, the diaphragm *D* is given a steady movement upward. The resulting photographic positive for the boundary of Figure 6, *a*, is shown in Figure 6, *b*, and is called the boundary pattern. The schlieren bands of *a* correspond to narrow vertical sections of the complete pattern. For publication it is common practice to turn the pattern through 90°, as shown in Figure 6, *c*. If more than 1 boundary is present in the channel, each appears as a separate peak in the complete pattern (e.g., Fig. 4, *a*).

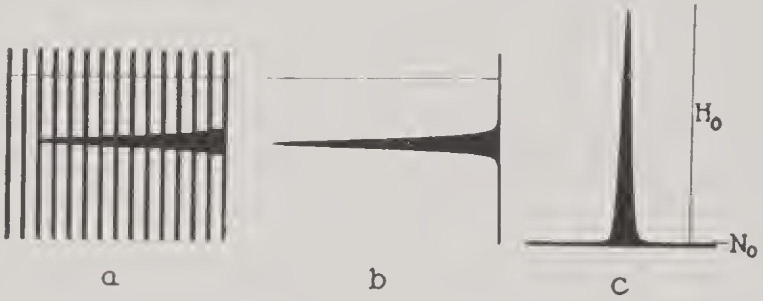

FIG. 6.—Schlieren band and scanning photographs of a single boundary.

As noted above, the edges of a schlieren band are conjugate to the 2 levels in the boundary at which the gradient deflects the light the same distance, ΔY, below the normal slit image. To a very close approximation this deflection is given by the relation

$$\Delta Y = ab\, \mathrm{d}n/\mathrm{d}h \tag{1}$$

where a is the dimension of the channel parallel to the optic axis, 25 mm in the Tiselius cell, and b is the optical distance from the center of the channel to the schlieren diaphragm. Consequently the outline of the shaded area in Figure 6, *c*, is a plot of the refractive index gradient as a function of the height in the channel. If E_c is the enlargement factor for the camera, the scale of abscissas for this plot is $E_c h$, which will be designated as H. The ordinate scale is, from equation (1), $a\ b\ M\ \mathrm{d}n/\mathrm{d}h$, designated as N, where M is the ratio of the rates at which the plate and diaphragm move in the scanning process. The vertical line at H_0, Figure 6, *c*, is due to a graduation ruled on the channel window with a diamond pencil and is taken as the origin of abscissas. The origin of ordinates is the horizontal line at N_0 joining those portions of the pattern outline that correspond to layers in the channel in which the solution is homogeneous. This is the base line of the pattern. In the ideal case this line is straight and horizontal. Actually, however, it is often distorted by imperfections in the lenses and in the cell and thermostat windows. Allowance for such distortion is made (described on p. 80) with the aid of a scanning photograph of the channel when it contains a homogeneous solution. Although it is a rather common practice, the recording, simultaneously with the boundary pattern, of a "base line" with light that has not traversed the cell cannot be recommended.

In addition to the scanning modification of the schlieren method for recording the pattern, there is the cylindrical lens procedure of Philpot (41) and Svensson (47). Since a clear account of this method is given by Pickels (this volume, p. 114) a description of its adaptation to electrophoresis will not be necessary here.

Limitations of schlieren methods.—Although both the scanning and cylindrical lens procedures give the complete pattern, they should be considered as supplementary to the schlieren band method originally used by Tiselius. Only by observing the distortion of the edges of these bands as the diaphragm approaches the normal slit image is it possible to detect the mild convection that frequently precedes more serious disturbances. In the scanning process the image mask screens all but the central part of the channel, whereas with the cylindrical lens the recorded value of the gradient is an average over the entire cross-section of the channel. Our preference is for equipment that is flexible with respect to the optical method employed so that the advantages of each may be utilized. In work with unfamiliar proteins or buffers the weak gradients at the edges of a boundary are examined at intervals during the experiment. If the edges of the schlieren bands have "horns" at the walls or a "chimney" at the center these effects can frequently be eliminated by reducing the current.

Although the phase-shifting schlieren diaphragm of Wolter (58) holds promise for the elimination of the diffraction effects arising at this barrier, the precision of the schlieren method is now limited by these effects. Owing to this diffraction the blackening on the photographic plate changes more or less gradually at the edge of the pattern. In reproducing the patterns of this chapter with "line" cuts, the effect has been lost. On the original plates, however, the successive diffraction maxima and minima in the illuminated region parallel to the edge of the pattern, except at the base line and top of a peak, are clearly visible. This is shown in Figure 7, which is a microphotometer trace through an edge (26). It is essential to expose and develop the plate so as to have a moderate density in the illuminated portions with the diffraction maxima clearly evident. On tracing the pattern one then follows a rather low density, the pencil line even penetrating slightly into the illuminated portion of the enlarged image of the plate, since the edge, in terms of geometrical optics, is at *H* in Figure 7. As shown in Figure 12, a pattern traced in this manner then agrees quantitatively with that given by an entirely different optical method.

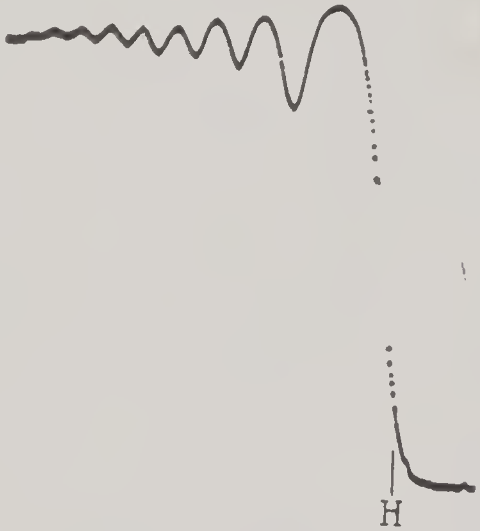

FIG. 7.—Microphotometer trace through edge of a boundary pattern showing effects of diffraction at the schlieren diaphragm.

Light sources.—The best patterns are obtained with monochromatic light, e.g., that given by the G.E. AH 4 mercury vapor lamp and a yellow filter, Wratten no. 22, or a green filter, Wratten no. 77A, and panchromatic plates of high contrast, e.g., Eastman CTC (Contrast Thin Coated). If this lamp illuminates the source slit without the aid of a condensing lens, it must be placed close to that slit and carefully adjusted to fill the schlieren lens uniformly with light.

In some instances materials that absorb or scatter light are present below certain boundaries in the channel and lead to underexposure of the conjugate portions of the pattern. If the effect is due to absorption, the light should be of a wavelength that is transmitted, e.g., red in the case of hemoglobin. If due to scattering, e.g., lipemic serum, every effort should be made to clarify the solution, by high speed centrifugation, say, before electrophoresis. This is not always practicable, however, in which case the effects of the scattering can be reduced by using red, or infra-red, light from an incandescent source (55) and a fast, red-sensitive emulsion of wide latitude. Although there is some reflection from the glass envelope, a useful lamp for this purpose is the Westinghouse 3.5 amp, 10 v galvanometer lamp, the linear filament of which is focused directly by the schlieren lens. Alternative procedures are to make 2 exposures, one of the correct density for the scattering zone, the other for the clear zone, or to use a bar as schlieren diaphragm.

Schlieren diaphragms.—Although a straight edge served as the schlieren diaphragm in obtaining the patterns of this chapter, a slit or a bar may also be used for this purpose in either the scanning or the cylindrical lens procedure. A slit gives the pattern as a bright band on a dark background, whereas in the case of a bar the background is light and the band dark. The bars and slits in use, however, are of such width as to act essentially as 2 parallel straight edges, each of which produces diffraction at the plate similar to that shown in Figure 7. The resulting band is generally so broad that one must trace and planimeter both edges. In the patterns of reasonably clear solutions, both edges give the same result and the additional work is hardly justified. If, as a result of absorption or scattering, the results from the 2 edges differ, the mean values should be used.

Schlieren optics.—For the pattern to record accurately the magnitudes and positions of the gradients in the channel, the optical system should be of good quality. In the case of the schlieren lens the requirements are similar to those of a telescope objective. Since this lens must have a diameter of 4 in. to cover the Tiselius cell, one having more than 2 elements, i.e., an achromat, is hardly practicable. In the case of an achromat, however, the image quality deteriorates at apertures much greater than F/9 so that a 4-in. achromat should have a focal length of 36 in. or greater. This consideration largely determines the dimensions of the electrophoresis equipment. Thus if both the schlieren and camera lenses of Figure 5 are used at unit magnification, the over-all length of the system is at least 18 ft.

Several expedients have been adopted to make the equipment more compact. One is to use a schlieren lens on each side of the thermostat so that the light through the cell is parallel (37). This device also eliminates a small error in the recording of sharp boundaries near the bottom or top of the channel due to the convergence of the rays from a single schlieren lens. Another is to reduce the diameter of the schlieren lens so as to cover the so-called microcells but not the standard one. Mirrors are also used, not to shorten the optical path but to reflect the light into a more compact space.

Focusing.—The effects of improper focus (27) of the source slit and of the channel are illustrated by a typical boundary in Figure 8. The pattern of *a* was obtained with both slit and cell correctly focused, a white line being drawn through the maximum gradient parallel to the reference line in order to demonstrate the symmetry of the gradients in the boundary. *b* was obtained with the cell moved 10 cm toward the camera, a shift from the object plane that causes the boundary to appear to be skew. The lower maximum in *b* is due largely to the shortened distance from the cell to the schlieren diaphragm. After returning the cell to the object plane and then moving the source slit 10 cm toward the thermostat the pattern of *c* was recorded. This displacement of the undeviated slit image from the plane of the schlieren diaphragm results in a sloping base line.

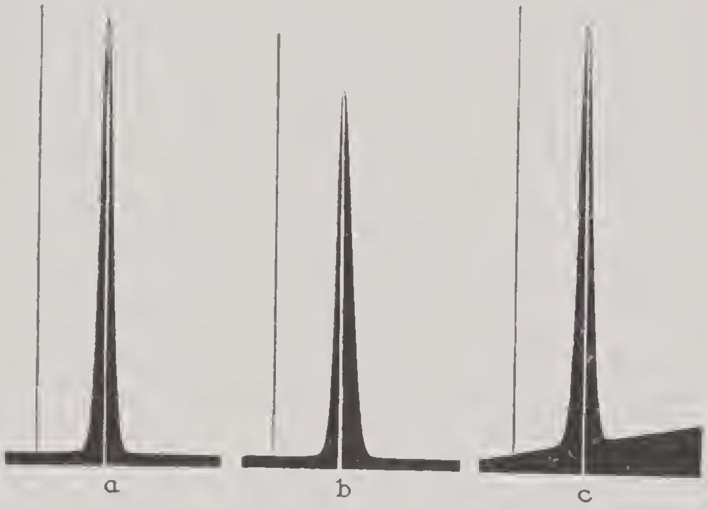

FIG. 8.—Effect of improper focusing of camera (*b*) and illuminated slit (*c*)

Although the slope of the base line affords a sensitive test for the focus of the source slit, the skewing of the boundary pattern is relatively insensitive to the camera focus. The object plane of the camera can be found most readily with the aid of a glass scale* placed in the thermostat normal to the optic axis. A low power microscope is then focused on the image of a scale line on the ground glass of the camera, the ground glass removed and the scale moved along the axis until the line image, as observed directly in the microscope, is most sharply defined.

Testing the optics.—An impression of the quality of the optical system may be obtained by the following means. With the thermostat windows removed the vertical dimension of the source slit is set at 0.05 mm, say, with the aid of a feeler gauge and the edge of the schlieren diaphragm placed just below the image of this slit. On the ground glass the image of the schlieren lens should now be uniformly illuminated at the same intensity as with the diaphragm removed. At unit magnification a perfect image of the source slit would also be 0.05 mm wide, and raising the diaphragm by this amount would cause the image on the screen to go uniformly dark. With good equipment the image goes dark with a diaphragm displacement of 0.06–0.08 mm, but seldom completely uniformly. Repetition of this test with the windows in place, then with water in the bath at 0.5 C and, finally, with the cell, also filled with water, in position in the bath affords information as to the contribution of each of these elements to the total distortion. Suggestions as to the significance of the shadows that appear on the screen as the slit image is intercepted are given in reference (27) together with additional procedures for aligning and focusing the equipment.

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* Precision glass scales, ruled for 10 cm at 0.5 mm intervals, are available from Lyman Nichols, 111 Vreeland Ave., Nutley 10, N. J.

III. Interference Methods

Rayleigh fringes.—Measurements of refractive index with the aid of methods based on the interference of light have long enjoyed a well deserved reputation for sensitivity and precision. Only recently, however, have such methods been introduced into moving boundary studies. The one that can be adapted to much of the existing schlieren equipment with only minor changes is a modification of the Rayleigh interferometer suggested by Philpot and Cook (42) and by Svensson (50). One form that this method takes is shown in Figure 9. Here *A* is a point source of monochromatic light, *L* is a lens that forms an image of this source in the plane at *P*, *M* is a plate with vertical slits that mask a boundary channel and a neighboring reference channel, respectively, and *C* is a cylinder lens, with its axis horizontal, that focuses the cell (not shown) vertically but does not affect the horizontal spacing of the Rayleigh fringes *F* that are formed at *P* by the vertical slits. If the fluid in the boundary channel is homogeneous these fringes are vertical, as shown in Figure 9. With 1 or more boundaries in this channel, however, they are warped, as shown in the photomicrograph of Figure 10, into overlapping segments of the complete refractive index-height curve that intersect diagonally the central diffraction envelope (30). Owing to the relatively great

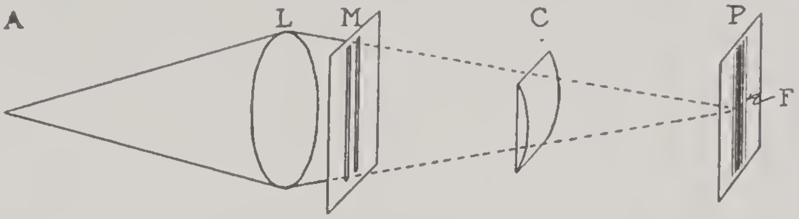

Fig. 9.—Modified Rayleigh interference method for obtaining the refractive index as a function of the height in the channel.

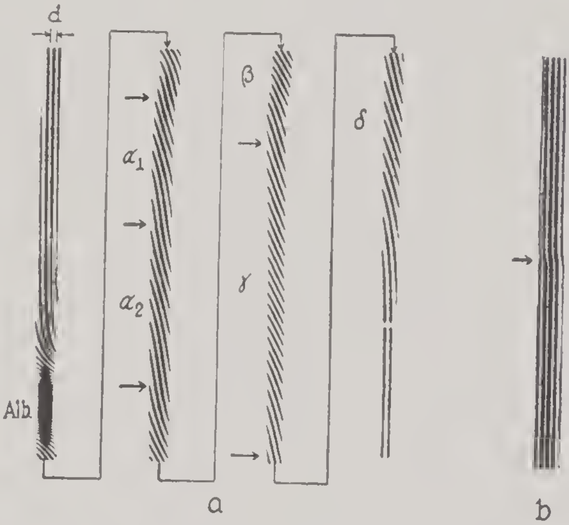

Fig. 10.—Photomicrograph of Rayleigh fringes obtained on electrophoresis *a*, a human serum (1 vol serum + 5 vol buffer). Here the fringe pattern of the rising boundaries has been enlarged in 4 sections, the crowding of the fringes conjugate to the albumin peak being evident in the left-hand part. Arrows indicate levels in the channel at which the vertical separations between the fringes are maximum. These levels correspond to the minima in the conventional schlieren pattern of the refractive index *gradients* in the channel. *b* a 0.001M KCl–0.00064M KIO_3 boundary. Here the total difference of refractive index across the boundary at the level indicated by the arrow is 77×10^{-7}. Lateral displacement of a fringe at this boundary is $60 \pm 1\ \mu$. Since the fringe separation *d* of Figure 10, *a*, is 169 μ, an uncertainty of 1 or 2 μ in the measurement of this displacement corresponds to a path difference of 0.01 wave. With green light of wavelength 5461 A, and the 2.5 cm thickness of the Tiselius cell, 0.01 wave is equal to 2×10^{-7} in refractive index.

length of the fringe negative, it was necessary to enlarge it in the 4 sections shown here.

The lens *L* of Figure 9 will be recognized as the schlieren lens and the plane *P* as that of the schlieren diaphragm, which may be replaced by a small plate holder. The cylinder lens of the Philpot-Svensson camera may be used at *C*. It is also necessary to replace the conventional mask of the Tiselius cell, which contains a slit in front of each side of the channel and a flap exposing 1 side at a time, by one having a double slit for each side. One of these exposes the channel and the other the reference path through the adjacent bath water. Each slit is 1.5 mm wide and the distance, δ, between their centers is 6 mm. To obtain interference, the reference path must contain a thickness of glass equal to that of the channel windows. This is done by fusing duplicates of the windows to the outside wall of each channel, a modification of the standard cell that is now available.† The "point" source may be obtained by superimposing a vertical slit upon the horizontal one used in the schlieren method. To obtain sharp fringes, the lateral dimension of this source should not exceed $^1/_4$ of the fringe separation, d of Figure 10. With an optical distance, b', of 186 cm from the double slit to the plate, $d = b'\lambda/\delta = 169\ \mu$ at the wavelength $\lambda = 5461$ A of the mercury green line. The vertical dimension of the source is not critical. In fact, if the cylinder lens is of sufficiently good quality a satisfactory source is obtained simply by rotating the schlieren source slit to the vertical position.

If the schlieren equipment is provided with a Philpot-Svensson camera the Rayleigh fringes can also be formed in its focal plane with even fewer changes than those mentioned above (50). Aside from the modified Tiselius cell with the double slit mask for each channel, the only change required is the rotation of the source slit to the vertical position. With this arrangement the adjustment of the axis of the cylinder lens to the vertical is quite critical and is facilitated by first obtaining the fringes with a point source and then refining the adjustment as the vertical dimension of the source slit is increased.

As in the case of the schlieren method, a "base line" may be recorded by photographing the fringes with a homogeneous solution in the channel. The alignment of a fringe pattern in the comparator (described on p. 83), is facilitated if the cell mask includes another pair of vertical slits in the path of which a properly oriented glass plate is placed. A set of vertical fringes is then formed at P, Figure 9, that is parallel to, but displaced from, the envelope of those from the boundary channel. This set thus affords an invariant frame of reference for each exposure.

Jamin fringes.—In the 2 micro-electrophoresis apparatus now available commercially (see Table 3) the principle of the Jamin interferometer has been utilized. In the Kern instrument (21, 31) monochromatic light is used and the image of the channel is crossed by a series of horizontal fringes at positions conjugate to the successive levels at which the optical path differs by 1 wavelength. A typical photograph of a human serum is reproduced in Figure 11 where the curve is that obtained by plotting the fringe number as ordinate against its position as abscissas. The points of inflection in this refractive index-height curve correspond to minima in the gradient curve and the data may be treated as described for Rayleigh fringes on page 84. The warping of the fringes near the walls that is apparent in Figure 11 may be the result of electro-osmosis, or of both this and thermal convection since the channel of this instrument is not thermostated. It should be noted, however, that this photograph is a 2.5 × enlargement of the actual channel, the clarity of the fringes near the wall indicating the wealth of detail available with this optical system.

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† Pyrocell Manufacturing Co., 207 East 84th St., New York 28, N. Y.

In the Antweiler instrument (5) the boundary and reference channels are "scanned" with coherent beams of white light of small cross-section, 0.5 × 0.05 mm. With the beams entering the top of the channels the white, zero-order fringe is brought into coincidence with the cross-hairs in an eyepiece by tilting the second Jamin plate with the aid of a graduated drum and screw. The platform carrying the optical unit is now lowered 0.1 mm, the white fringe again centered on the cross-hairs and the new drum reading recorded. This process is repeated until the 12 mm height of the channel has been covered. Since the drum is calibrated to give the path difference between the 2 channels, the 120 data can thus be plotted to give a refractive index-height curve like that of Figure 11.

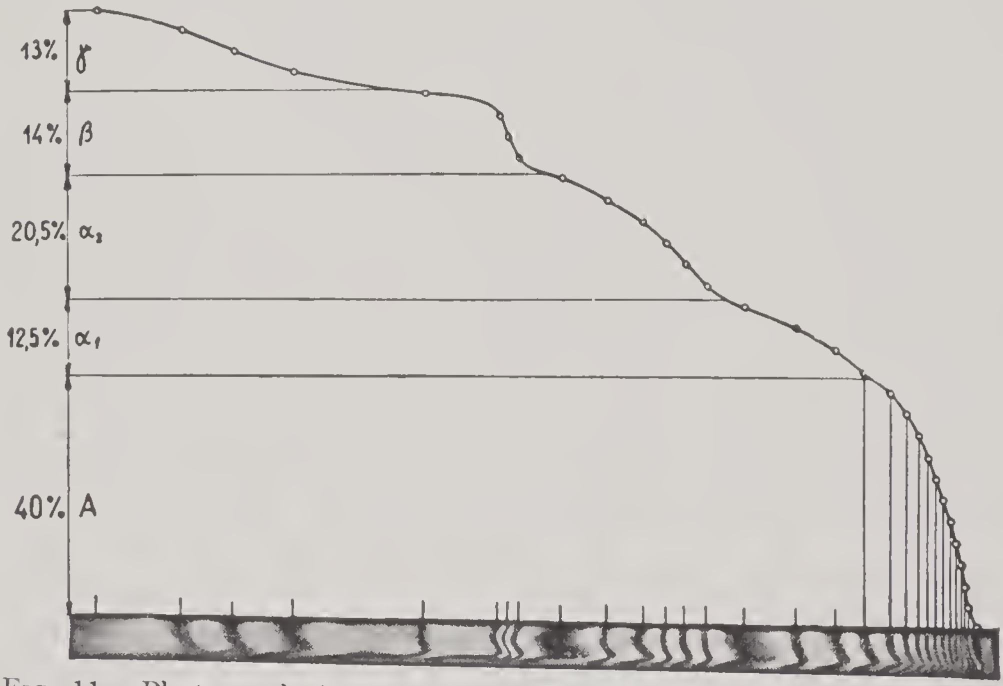


FIG. 11.—Photograph (enlarged 2.5×) of the Jamin fringes of a normal human serum in the Kern micro-electrophoresis apparatus, together with the refractive index-height curve derived therefrom. (Reproduced through courtesy of the Kern Co., 5 Beekman St., New York, 38.)

Evaluation.—In their present state of development interference methods are not as satisfactory as the schlieren procedures for the determination of boundary position, i.e., mobility measurements. For analyses, however, they appear to be more precise. This doubtless arises from the greater accuracy with which the fringes can be located as compared with the edges of the relatively coarse diffraction patterns that are encountered in using the schlieren method. The fringe displacement at a sharp boundary such as that of Figure 10, *b*, can be measured to within 1 or 2 μ. With $d = 169\ \mu$ this corresponds to a path difference of 0.01 wave. With $\lambda = 5461$ and $a = 2.5$ cm, 0.01 wave is equal to 2×10^{-7} in the refractive index.

IV. Analysis of the Pattern

Pattern areas.—For the determination of the areas enclosed by the peaks in a pattern such as that of Figure 4, *a*, the photographic plate is placed in an enlarger and the projected image of the pattern traced on a sheet of paper (Fig. 4, *d*). This sheet is then shifted until the *lower* edge of the base line exposure (Fig. 4, *b*), is brought into coincidence with the horizontal edges of the pattern conjugate to those portions of the channel in which there are no gradients. Tracing of the lower edge of *b* then gives the base line. If gradients are present throughout the channel it is necessary to interpolate on the pattern tracing for the same diaphragm setting, N_1, as that at which the base line recording began and then shift the paper to bring the image of the *upper* edge of *b* to this level before tracing its lower edge. Similarly, by shifting the sheet again so that the image of the graduation at H_0 in *c* is coincident with this line in the tracing of *a*, the initial boundary position can be recorded as shown at H_i in *d*.

As is evident in Figure *d*, there is appreciable overlapping of the gradients of adjacent boundaries and the division of the area is somewhat arbitrary. Following the procedure of Tiselius and Kabat (54), this is done by drawing an ordinate from the lowest point between adjacent peaks. With the aid of a polar planimeter, e.g., Keufel and Esser no. 4242, the area under each peak is determined in arbitrary units. In this determination it is essential that the graduated wheel of the planimeter move on a uniformly smooth surface. Since the total area of a pattern is proportional to the difference in refractive index of the protein and buffer solutions that met at the original boundary, it should be the same for the patterns from the 2 sides of the channel in a given experiment.

If the plasma proteins are assumed to have the same specific refraction and the apparent relative composition is all that is required, the ratio of the area due to the albumin boundary, say, to the total area of the pattern exclusive of that due to the concentration boundary, i.e., the δ or ϵ effect, is the fraction of the total protein present as albumin. See, for example, the last line of Table 4.

If the difference, Δn_i, of refractive index across a boundary due to the ith component is required, the relation is

$$\Delta n_i = A_i/abGE_cE_tP \tag{2}$$

in which the terms not previously defined are: A_i = area of i-peak in planimeter units; G = ratio of plate to diaphragm travel in the scanning process or tangent of the angle of the inclined slit with the vertical in the Philpot-Svensson camera; E_t = tracing enlargement, and P = planimeter units/cm². If only 1 protein is present and its refractive increment k is known (40), the total area of the pattern affords, with the aid of equation (2), an accurate measure of its concentration p, since $p = \Delta n/k$.

TABLE 1.—DETERMINATION OF THE OPTICAL DISTANCE, b

ELEMENT	Δx	n	$\Delta x/n$
$a/2$	1.25	1.334	0.94
Cell window	0.30	1.526	0.20
Cell window to inside thermostat window	23.77	1.334	17.82
Inside thermostat window	0.63	1.526	0.42
Air space in double window	2.54	1.000	2.54
Outside thermostat window	0.63	1.526	0.42
Outside thermostat window to diaphragm	159.72	1.000	159.72
			b = 182.06 cm

Channel depth.—The channel depth a is determined with the aid of a small brass bar whose length l is slightly less than a and whose ends are flat and parallel. With the channel in a horizontal position on the stage of a microscope the bar is inserted and the difference $a - l$ determined with the aid of the graduated fine adjustment by focusing alternately on the upper end of the bar and the inside wall of the top window. This is repeated as the bar is shifted along the channel.

The optical distance "b."—If there is no lens between the channel and the schlieren diaphragm, one method for determining b is illustrated by the data of Table 1 for a typical installation. In the 1st column is listed each successive element in the path, while its dimension, parallel to the optic axis, is given in the 2d column. The factor b is the sum of these distances after division of each by the corresponding refractive index (column 3) referred to air. If the light through the thermostat is accurately parallel, b is simply the focal length of the 2d schlieren lens and can frequently be obtained from the manufacturer. Since the principal planes of most achromats lie within the glass, the distance from the crown of the lens to the diaphragm is somewhat less than b. A small glass prism of appropriate and accurately known power in water at 0° C is, however, the most convenient accessory (27) for the determination of b. If such a prism is placed in the thermostat with the plane bisecting the prism angle coincident with the object plane of the schlieren camera, the resulting displacement of the slit image is a measure of b. This displacement may be obtained by noting the diaphragm positions at which the image of the prism and that of the remainder of the field go dark. It is also clear that b may be determined with the aid of equation (2) if a boundary is present in the channel across which the difference of refractive index is known. One formed between water and a 1 per cent solution of sucrose may be used if it is shifted into view sufficiently slowly. At 1° the refractive index increment for this solution is 1468×10^{-6} at a wavelength of 5461 A, i.e., the mercury green line (13).

Enlargement factors.—Both enlargement factors of equation (2), i.e., E_c and E_t, are conveniently determined with the aid of the glass scale. This is placed in the object plane of the schlieren camera and photographed, and the ratio of the scale interval in the negative to that in the real scale, as determined with the aid of a comparator, is E_c. To determine E_t the enlarged image of a contact print of the scale is projected onto the scale itself.

Computation of mobility.—The enlarged tracing of the pattern can also be used to determine the boundary displacements from which to compute mobilities. The initial boundary position is taken as the center of the schlieren band after interruption of the compensation but before passage of the current. As is clear from a comparison of Figure 4, a and c, it is incorrect to assume that the concentration boundary does not move on passage of the current and to use its position as that of the initial boundary.

If the gradient curve for the boundary after electrophoresis is symmetrical the midpoints of a series of chords will lie on a straight line parallel to that due to the graduation on the cell, and this is the boundary position. If, as in the case of the γ globulin peak in Figure 4, d, for example, the midpoints do not lie on such a line, the boundary is skew and its correct position is that of the first moment of the gradient curve (26). The location of this is tedious, however, and for many purposes it is adequate to take the boundary position as that of the ordinate that bisects the area under the gradient curve. This is determined by trial and error with the aid of the planimeter. If κ_p is the specific conductance of the protein solution and i amp is passed for t sec in causing the boundary to move through a distance of ΔH cm on the tracing, the corresponding displacement, Δh, in the channel is $\Delta H/E_cE_t$ and the mobility is

$$u = \frac{\Delta h \cdot s \cdot \kappa_p}{it} \tag{3}$$

where s is the average cross-sectional area of the channel.

Channel cross-section.—For the determination of s the assembled Tiselius cell is filled with mercury to a level somewhat above the junction of the center and top sections. Shifting the center section to 1 side then isolates its contents and permits pouring off of the excess mercury in the top section. The center section is next returned to its original position, but isolation of the mercury therein from that in the bottom section is retained by shifting this section simultaneously with the center one. Now, by pouring the contents of the 2 sides of the center section into 2 previously weighed beakers and reweighing, the total volume of each side may be computed with the aid of the density of the mercury at the temperature of the operation. An average value of s that is adequate for most work is the quotient of this volume and the height of the section as determined with the aid of a vernier caliper. This is an average, however, over the entire height, including the horizontal end-plates. The channels of most center sections have a reasonably uniform cross-section except where they pass through the slots in the end-plates. In careful work correction for this can be made by subtracting the volume of a channel in these plates from the total volume and dividing the difference by the total height less the thickness of the plates (3). The thickness, width and depth of a slot in an end-plate can be determined by means of a micrometer, feeler gauge and inside vernier caliper, respectively.

Fringe analysis.—For the analysis of interference fringe patterns such as that of Figure 10, *a*, the procedure depends upon the character of the "base line" fringes. If these are straight to within 0.1 of their separation d and the allowable error in n is 2×10^{-6}, i.e., 0.1 of a wave in the Tiselius cell, no correction is necessary. The pattern of the boundary system is then placed in a comparator with the vertical fringes conjugate to the homogeneous column of buffer at the top of the channel aligned parallel to the axis of that instrument. With the cross-hairs initially focused on a minimum, say, the reading, H, at each minimum, j, intersecting the envelope is tabulated. Since each fringe represents a refractive index increment of $\Delta n = \lambda/a$, a plot of these readings against the fringe number j gives that index as a function of the height, e.g., Figure 11. If, on the other hand, the reciprocal, $1/\Delta H$ of the vertical separations between the fringes are plotted as ordinates against their mean position $\bar{H}$ as abscissas the curve of refractive index gradient versus height is obtained. This is shown in Figure 12, where the circles indicate the values of $1/\Delta H$ for the fringes of Figure 10 and the full curve is a tracing of the schlieren scanning pattern obtained in the same experiment. From this figure it is clear that the 2 independent optical methods give the same result.

For an analysis it is unnecessary, however, to prepare a plot. The position of a minimum in the gradient curve corresponds to a maximum in the fringe separation, and the number j of the fringe at which it occurs can be determined by inspection of the differences ΔH, or to a fractional part of a fringe by numerical interpolation (30). Thus, if $\Delta H_1 = H_j - H_{j-1}$, $\Delta H_2 = H_{j+1} - H_j$, and $\Delta H_3 = H_{j+2} - H_{j+1}$ and ΔH_2 is a maximum, i.e., $\Delta H_1 < \Delta H_2 > \Delta H_3$, the value of j at the apex of the curve of ΔH versus j is

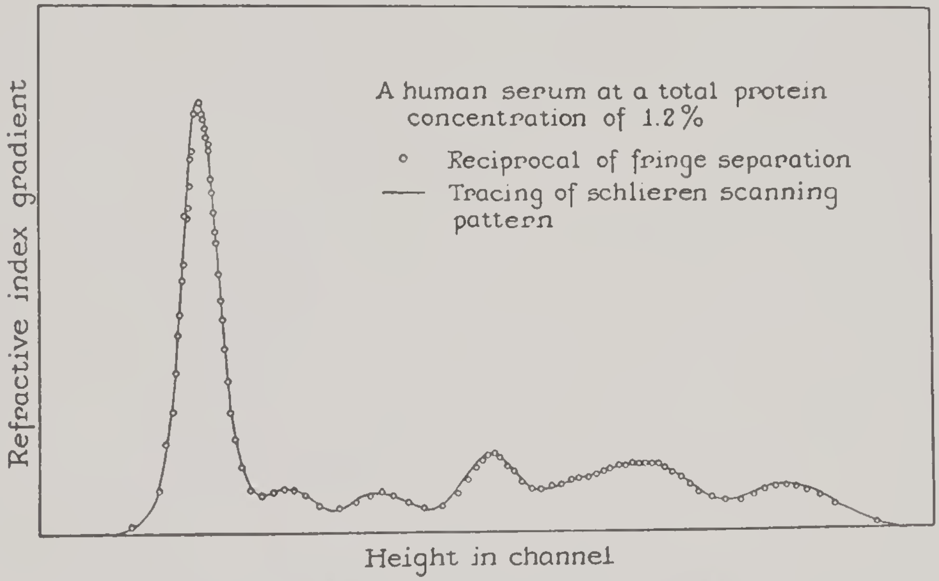


FIG. 12.—Pattern of a human serum (1.2 per cent total protein concn.) obtained by 2 independent optical methods.

$$j_m = \frac{(2j+1)\Delta H_2 - j\Delta H_3 - (j+1)\Delta H_1}{2\Delta H_2 - \Delta H_1 - \Delta H_3}$$

In Figure 10, for example, the values of $\Delta H_{1,2,3}$ at $j = 44$ are 466, 532 and 493 μ, respectively, and $j_m = 44.6$. In this figure maxima occur at $j = 0$, 44.6, 50.4, 56.7, 69.0, 92.0 and 102.0, this last interval representing the δ, i.e., protein concentration, boundary. The positions of these maxima are indicated by the arrows in Fig. 10, *a*. If all of the serum proteins have essentially the same specific refraction the apparent composition of this serum is 44.6/92.0 or 48.5 per cent albumin, 6.3 per cent α_1-, 6.8 per cent α_2-, 13.4 per cent β- and 25.0 per cent γ-globulin.

If the base line fringes are not straight a refractive index-height curve for the imperfect optical system must be constructed and subtracted from that with the boundaries present in the channel.

V. Interpretation of the Pattern

Ideal electrophoresis.—Within the pH-stability range of most proteins the mass/unit net charge, i.e., the equivalent weight, is large in comparison with that of the buffer ions. Thus a 1–2 per cent solution of protein usually corresponds to a very low equivalent concentration of this material although it makes a major contribution to the density and refractive index. The conductance and pH, on the other hand, are determined largely by the buffer ions and, if these are present in sufficient concentration, provide, on passage of the current, a uniform electric field in which the protein ions drift. In the ideal case of low protein and high salt concentration there are then no superimposed gradients of other species at a boundary across which a protein disappears, and the patterns from the 2 sides of the channel are mirror images of each other, i.e., they are "enantiographic."

The evaluation of the *apparent* composition and mobilities described in the previous section assumes that this ideal situation is realized in practice. Actually it is not, the departure from the ideal in the case of real patterns being evident from the presence of the concentration boundaries and from the fact that the volume through which a boundary rises on passage of the current is generally greater than the displacement of the corresponding descending boundary. Moreover, the area due to a rising boundary is usually less than that of the corresponding descending boundary, compensation for the differences being made by the δ boundary whose area is greater than that of the ϵ effect. In the absence of immobile components the ϵ boundary is merely a change in the concentration of the buffer salts, and all ion species, both buffer and protein, remain in the same proportion across the δ boundary. Consequently the deviations from enantiography would not be a source of error if it were not for the fact that the disappearance of a protein species across a moving boundary is accompanied by adjustments in the concentrations of all other species that may be present. Moreover, these superimposed gradients are not proportional to that of the disappearing species and hence do not cancel in the evaluation of the apparent composition. Thus the question arises as to the extent the apparent composition differs from the true value.

Apparent and true composition.—Since no sufficiently precise measurements on protein mixtures of known composition have been made, a really satisfactory answer to this question is not available. The work of Perlmann and Kaufman (39) affords, however, an idea of the magnitude of the error. With the aid of a 0.1M sodium diethylbarbiturate buffer at pH 8.6 they found that the apparent albumin content of a human plasma, as determined from the patterns of the rising boundaries, varied linearly from 58.2 to 54.6 per cent as the total protein concentration was reduced from 2.66 to 1.00 per cent. In the case of the patterns of the descending boundaries the variation was less, being almost within the limits of error of the measurements, but the results from both sets of patterns extrapolated to the presumably correct value of 53 per cent albumin at zero concentration of protein.

The error also depends upon the nature of the buffer solvent, diethylbarbiturate being one of the best in this regard (28). Dole's theory (9) has been useful in indicating the magnitude of the error to be expected in a given medium and is in qualitative accord with experiment. However, this theory does not take into account the effect of the small pH changes across the boundaries and in many cases, including serum analyses, indicates larger differences between the true and the apparent composition than probably exist.

Both theory and experiment indicate that the apparent values obtained from the pattern of the descending boundaries are more nearly correct than those from the rising boundaries. This is true of both the composition and the mobilities. In fact, the mobility computed from the displacement of the fastest descending boundary is correct since here a species, albumin in the case of serum at pH 8.6, disappears from a solution, the protein solution, whose conductance is known. Across all other moving boundaries in the system a species disappears from a solution that has been created by the electrophoresis and in which the conductance differs slightly from that of the original protein solution (51).

Mobility vs. pH.—In view of the foregoing considerations the pH-mobility curve of a protein, and hence its isoelectric pH, should be determined, if possible, upon samples of the isolated material. Moreover, concentrations of not more than a few tenths of a per cent should be used in order to minimize the possible effect of viscosity upon the result. This is especially true in the case of materials such as hyaluronic and the nucleic acids whose solutions are quite viscous.

In addition to the departures from ideal behavior mentioned above there are those encountered with mixtures in which interaction between the components occurs. Although the consideration of such systems is beyond the scope of this chapter, references (4), (25) and (45) are to work in this field.

VI. Preparation of Solutions

Buffers.—In electrophoresis it is frequently necessary to prepare a variety of buffer solutions and it is well for the investigator to be familiar with the most commonly used systems. These are listed in the 1st column of Table 2 in the order of their pK′ values as given in the 2d column. The pK′ of a buffer system is the pH of a half-neutralized solution of the weak buffer acid, or base, at a given ionic strength. In Table 2 the values of column 2 are for an ionic strength, μ, of 0.1 and a temperature of 25 C. The classification of the buffers, as listed in column 5, is on the basis of the charge type of the acid form as suggested by Brønsted (7). If the buffer is of the uncharged- or anionic-acid type the pH of a given solution increases slightly with dilution, as is indicated by the pK values at zero concentration, column 3.

TABLE 2.—BUFFERS FOR USE IN ELECTROPHORESIS

1 Buffer System	2 pK′ μ = 0.1 25 C	3 pK μ = 0 25 C	4 pK μ = 0 0 C	5 Acid Type	6 References
Glycine, HCl	2.50	2.35	2.45*	cationic	14, 16
β-alanine, HCl	. . .	3.55	3.66	cationic	34
Acetic acid, NaOH	4.64	4.76	4.78	uncharged	14
NaH_2PO_4, Na_2HPO_4	6.85	7.21	7.30*	anionic	14, 15
Diethylbarbituric acid, NaOH	7.82†			uncharged	35
Glycine, NaOH	9.67	9.78	10.52*	uncharged	14, 16
β-alanine, NaOH	. . .	10.23	11.00	uncharged	34

* Extrapolated. † pK′ (0°) − pK′ (25°) ≃ 0.36.

Since it is inconvenient to thermostat the glass electrode and vacuum tube potentiometer used for most pH measurements, these are generally obtained at a room temperature of 20 to 25 C. In the case of a buffer whose pH is less than 7 the value thus obtained is not very different from that at 0 C, the temperature of the electrophoresis. Alkaline buffers, on the other hand, have appreciable temperature coefficients. This is shown in Table 2 where the change in pK from 25 to 0 C, columns 3 and 4, is a close approximation to the shift in pH over this temperature interval. Thus a glycine buffer of pH 9.60 at 25 C will have a pH of 10.35 at 0 C.

Owing to the hygroscopic nature of many buffer salts it is generally best to prepare the solution from standard solutions of a strong acid, or alkali, and the weak buffer electrolyte. In the case of uncharged acid systems, B moles of sodium hydroxide are added to A moles of the weak acid, where $A > B$. As a close approximation the pH is given by the relation

$$\mathrm{pH} = \mathrm{pK}' + \log \frac{B}{A - B} = \mathrm{pK}' + \log \frac{(\text{salt})}{(\text{acid})} \tag{4}$$

and the ionic strength is the concentration of the sodium ion if this is large in comparison with that of the hydrogen ion. Thus, a solution containing 0.1 mole of sodium acetate and 0.01 mole of acetic acid per liter is 0.1M and 0.01M in these 2 constituents, respectively, and has a

$$\mathrm{pH} = 4.64 + \log \frac{0.1}{0.01} = 5.64$$

and an ionic strength of 0.1. As another example, suppose the composition of a 0.1 μ glycine buffer of pH 10.00 is required. Equation (4) is rearranged to

$$\frac{(\text{salt})}{(\text{acid})} = \text{antilog}\ (\mathrm{pH} - \mathrm{pK}') = \text{antilog}\ 0.33 = 2.14$$

Since (salt) = 0.1 the acid concentration must be 0.0467 and would require, per liter of solution, 0.1 mole of sodium hydroxide and 0.1467 mole of glycine.

In the case of phosphate buffers equation (4) may be used if (acid) = $M_{\mathrm{NaH_2PO_4}}$ and (salt) = $M_{\mathrm{Na_2HPO_4}}$ but the ionic strength is $\mu = M_{\mathrm{NaH_2PO_4}} + 3M_{\mathrm{Na_2HPO_4}}$. All of the other buffers of Table 2 are monovalent and afford a maximum buffer capacity for a given ionic strength.

Selecting a buffer.—Conspicuous by their absence from Table 2 are monovalent buffers for the physiologically important range from pH 5 to 8. Several systems, e.g., cacodylic acid and the substituted phenols, are available but must be used with caution because of their tendency to denature proteins. Others, such as the substituted glyoxalines (20), are not readily available. Still others, e.g. formic, lactic, citric, phthalic and boric acids, are omitted because of their tendency to form complexes with proteins, although it cannot be presumed that those of Table 2 are entirely inert in this respect.

In most applications a buffer system is used to cover a pH range of about 1 unit on either side of its pK′ value. In electrophoresis, however, the effect of high concentrations of the weak buffer electrolyte upon the temperature of maximum density of the solution should be considered, as is shown later (p. 95). Thus a 0.1 μ β alanine buffer at pH 3.64 would be preferable to an acetate buffer of the same pH and ionic strength since the concentration of acetic acid would be 1M. Another consideration in the choice of a buffer is the extent to which the resulting patterns of the rising and descending boundaries are enantiographic. If the proteins are present as anions, enantiography is favored by the use of a buffer of the uncharged acid type; if present as cations, a cationic acid type is preferred (28). A high buffer capacity is not always essential, however, and it is sometimes desirable, especially in obtaining pH-mobility curves, to use low concentrations of the buffer electrolytes and build up the ionic strength by addition of a neutral salt such as sodium chloride.

Stock solutions.—Convenient stock solutions for use in the preparation of buffers are 2M sodium hydroxide, 2M acetic acid and 2M hydrochloric acid. Since it determines the ionic strength in many instances, the 2M NaOH is the most important of these reagents and is prepared as follows. One lb of reagent grade NaOH (from sodium) is dissolved in an equal weight of water. On standing the sodium carbonate present as impurity, being insoluble in this 50 per cent solution, settles and the clear supernate is decanted into a stoppered, paraffin-lined flask. The density of this solution is 1.525, and 117 ml is required for 1 l of 2 + M solution. The exact concentration is determined by titration against potassium acid phthalate with phenolphthalein as indicator and is then adjusted to 2M ± 0.2 per cent by addition of the computed volume of water. A 2d titration checks this adjustment. If each sample of $KHC_8H_4O_6$ is 4 g, or slightly less, a 10 ml buret is adequate for the required alkali and a precision of 0.02 ml is readily attained. This solution is then used to standardize those of acetic and hydrochloric acid.

Stock solutions of glycine, alanine and the phosphates can also be prepared, by direct weighing of the solutes, but since these can support the growth of molds they should be used promptly. Owing to its limited solubility diethylbarbituric acid (HV) is used in the solid form. In fact, the preparation of the 0.1M NaV–0.02M HV buffer is expedited if the mixture of acid and alkali plus most of the water is heated in the volumetric flask until the acid is dissolved before cooling and filling to the mark.

Potassium acid phthalate is also used for the calibration of the pH meter, a 0.05M solution having a pH of 4.00 at room temperature. This meter may also be checked occasionally at other pH values. One secondary standard (15) is 0.05M $Na_2B_4O_7 \cdot 10H_2O$ with a pH of 9.18 at 25 C. Another is 0.1M HCl with a pH of 1.08 at 25 C.

Dialysis.—As noted on page 63, the protein solution is prepared by dialysis against the buffer solution. This is done with the aid of a bag made of Visking cellulose tubing, a long one of small bore being preferred. For example, after an 8 in. section of $^{18}/_{32}$ in. tubing is made pliable by soaking in water, one end may be twisted and a knot tied therein. After testing for leaks by filling the bag with water under gentle pressure it is emptied and 12–15 ml of the protein solution introduced, together with a glass bead. The bag is then closed by tieing a knot in the upper end, after which it is suspended in about 250 ml buffer in a stoppered, footed cylinder and placed in a refrigerator. Inversion of the cylinder a few times during the 12–24 hr period required for attainment of the Donnan equilibrium provides some agitation. The buffer in the cylinder is replaced by a fresh portion for a 2d period, after which the bag is transferred to the remainder of the buffer solution, 1.5 l if a 2 l sample was prepared originally, for a 3d period of 12–24 hr. This buffer dialyzate is then used in forming the boundaries and for filling the electrode vessels.

Although the foregoing dialysis procedure eliminates virtually all diffusible solutes except the buffer electrolytes, this is not always essential. Dialysis overnight against the 1.5 l of buffer required for the cell and electrode vessels is frequently adequate since the foreign ions are then present on both sides of the initial boundaries at low concentrations and do not give rise to moving salt boundaries of appreciable magnitude. The time can be reduced by dialysis (43) at room temperature. Continuous agitation also hastens the process somewhat, although the rate appears to depend mainly on diffusion through the cellophane. Most tubing has a wall thickness of 0.02 mm when dry, and material having walls thicker than this should be avoided.

Serum or plasma is usually diluted to a total protein concentration of 1–2 per cent for electrophoresis. This may be done either before or after dialysis, the diluent in the first case being the buffer whereas in the second case it is the buffer dialysate. Serums or plasmas that have been diluted with the buffer but not dialyzed against it should not be used. As a result of the diffusion of water the concentration of protein in the bag changes slightly during dialysis, a fact that is sometimes overlooked.

VII. Measurement of the Conductivity

Conductivity cell.—The conductance of the protein solution may be determined with the aid of the shielded bridge and conductivity cell developed by Shedlovsky (44). The electrodes of the cell

(Fig. 13) are hollow truncated platinum cones *e–e*, the outer surfaces of which are sealed to the glass and make contact with mercury in the tubes *t–t* by means of platinum wires. The inner surfaces in contact with the solution are platinized (44). Owing to the relatively unimpeded flow when the cell is filled by means of the tubes *f–f*, there is little tendency for air bubbles to be trapped, an advantage in the case of protein solutions. The glass rod *r–r* affords mechanical strength and is used to support the cell in the 1.9 l Pyrex Thermos vessel #8621 that serves, when filled with shaved ice and distilled water, as a thermostat for the conductance measurement.

A cell of this type requires 3 ml of solution and has a constant, K, of about 16. This constant is determined with the aid of a solution of known conductance; e.g., one containing 7.4789 g of potassium chloride/1,000 g of water (values against brass weights in air of density 0.0012) has a specific conductance at 0 C of 0.0071379. If the resistance of the cell when filled with this solution is R' ohms, then $K = 0.0071379R'$. Now, if another solution has a resistance of R ohms, its specific conductance is $\kappa = K/R$.

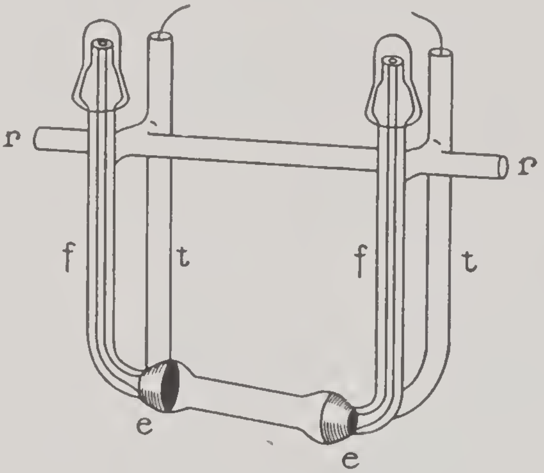


FIG. 13.—Shedlovsky conductivity cell.

A cell having a constant of 10–20 will have a resistance of a few thousand ohms when filled with most 0.1M buffers. In this range the bridge described below is most sensitive. For dilute solutions a cell having a constant of 1–2 may be used or that shown in Figure 13 may be shunted with a resistance having an accurately determined value R_1 of, say, 10,000 ohms. If R_2 is now the balancing resistance, that of the cell is $R = R_1R_2/(R_1 - R_2)$.

Conductivity bridge.—The conductivity bridges in use in this laboratory are built around the Leeds and Northrup ratio box no. 1153 and their 4-dial decade resistance box no. 4747. Since the ratio arms are fixed at 1,000 ohms each, the cell resistance, when balanced, is given directly to the nearest ohm by the setting of the decade dials and to the nearest 0.01 ohm by the graduated 1.1 ohm rheostat R in the ratio box. The wiring diagram of the associated oscillator and amplifier is shown in Figure 14. A frequency of about 2,000 cps is used and, by shielding the components independently as indicated by the dotted lines in the figure, it is possible to house the entire bridge in a 19 × 18 × $13^1/_2$ in. Par-metal cabinet without the amplifier picking up a signal directly from the oscillator.

The balancing procedure is as follows. With the switch s_2 thrown to D, the resistance arm and the capacities c_1 and c_2 are adjusted to give silence in the phones. The switch s_2 is then thrown to ground and silence is again obtained by balancing the earthing capacity CAP and the earthing resistance G of the ratio box. The capacity c_3 shunts that of the box and can also be used in making this balance. Now, with s_2 returned to D a new bridge balance is found. If necessary, this process is repeated until silence is obtained with s_2 either grounded or at D.

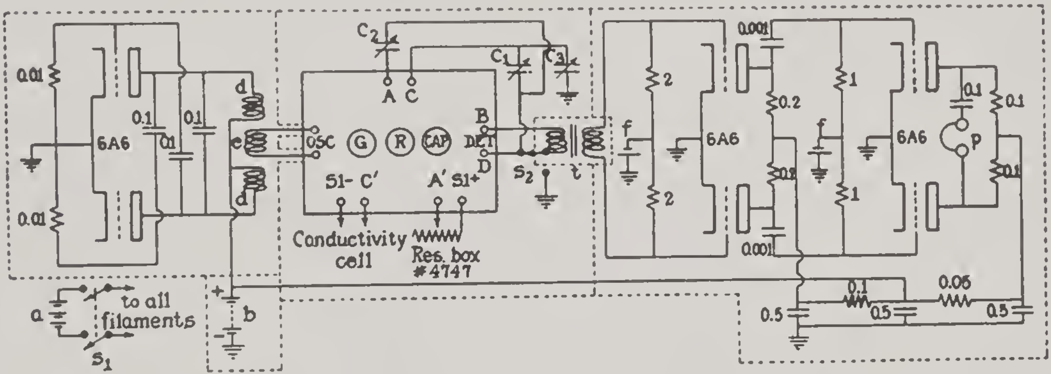


FIG. 14.—Conductivity bridge. All fixed resistors are 1 W; values in megohms. All fixed condensers are 400 v; values in microfarads. a = 6 v storage battery. b = 90 v "B" battery. c_1, c_2, c_3 = 30, 350, 350 $\mu\mu$f, respectively, variable air condensers. d = 30 millihenry radio frequency choke, Budd #CH1218. e = 60 millihenry radio frequency choke, Budd #CH1219. f = $1^1/_4$ v Mallory grid bias cell. p = crystal phones, Brush model A. s_1, s_2 = dpst and spdt switches. t = input transformer, General Radio 578A (detector terminals of the ratio box may also be connected directly to the amplifier, omitting this transformer).

Since the conductance is measured at 0° C, the mobility that is computed with the aid of equation (3) is the value at this temperature, although the boundary displacement occurs at a somewhat higher temperature. This is due to the fact that the conductance is sensitive to the temperature whereas the displacement changes only slightly.

It is also good practice to determine the conductance of the buffer dialysate since a comparison with that of the protein solution indicates the effectiveness of the dialysis, the values for the 2 solutions reaching a small but constant difference as the Donnan equilibrium is attained. Owing to the precision with which conductances can be determined, such a measurement affords a check on the care exercised in the preparation of the buffer solution.

VIII. Control of Convection

Convective circulation.—By convection is meant the circulation of solution in the channel that results from (*a*) the horizontal density gradients accompanying the dissipation of the heat that is generated by the passage of the current, i.e., the Joule heat, (*b*) electro-osmotic streaming, (*c*) the development of layers of solution less dense than those above or more dense than those underneath and (*d*) possible drainage effects due to the layer of immobilized solution at the wall of the channel. This last effect appears to be a source of convective disturbance only in the case of abnormally viscous solutions, such as those of the nucleic and hyaluronic acids, and is apparent in the distortion of the boundary that occurs on shifting it in the channel.

In electrophoresis in the Tiselius cell it is safe to assume that some circulation always occurs in the conducting, homogeneous columns of solution between the boundaries. The problem, then, is to choose conditions which reduce this circulation to such an extent that the vertical density gradients at the boundaries can stabilize them against the disturbing effects of the convection. The requirement for gravitational stability, namely, that the light solution must always be above the heavy one, represents a limitation to the Tiselius method that is not present in packed channels where the packing material provides interstices in which separations can occur without regard to this requirement. Although electrophoresis in packed channels, e.g., paper strip ionography (11), ionophoresis in silica- (8) and agar-gels (38) and other packings (49), is now being investigated for qualitative analyses and preparative purposes, it will not be treated here.

The β-globulin disturbance that is encountered in work with serum and plasma is probably an example of gravitational instability. At the descending β-globulin boundary localized convection frequently produces an abnormally sharp "spike" in the pattern.

Effect of channel width.—The role of electro-osmosis in moving boundary work is not yet clear. In 1940 the late Mr. Klett kindly furnished the author 2 center sections identical with *II* of Figure 1, *a*, except that the channel widths were 1.5 and 0.85 mm, respectively. In each of the 3 channels thus made available, a 1.16 per cent solution of ovalbumin in a 0.1 μ sodium phosphate buffer at pH 6.8 was allowed to migrate about 6.7 cm at an electric field strength E of 7.5, and also at 15 v/cm. The essential portion of each pattern of the rising boundaries is shown in Figure 15. Since the best pattern was obtained under the conditions usually employed, namely, in the Tiselius cell at the lower field strength, further work with the narrow channels was discontinued. In view, however, of the recent introduction of microcells with channel widths as low as 1 mm, the patterns of Figure 15 acquire a new interest.

The most striking feature of the patterns is the progressive deterioration, at both field strengths, of the resolution of the A_1, A_2 and A_3 components of ovalbumin as the width of the channel is decreased. Moreover, in each channel the maximum refractive index gradient is lower at $E = 15$ than at $E = 7.5$. Although electroosmotic streaming may be a factor in the narrow channels, it does not appear to be directly responsible for the inferior resolution since the "stationary" concentration boundary in each experiment, except in the Tiselius cell at $E = 15$, was unaffected. When a boundary is shifted at a given rate by means of the compensator it spreads more rapidly in a narrow channel than in a wide one, due possibly to relatively slow "drainage" of the immobilized solution in contact with the wall. Although a boundary moves in an electric field as a result of ion migration, and does not involve displacement of the solution in bulk, as during compensation, the effects on the resolution appear, superficially at least, to be similar.

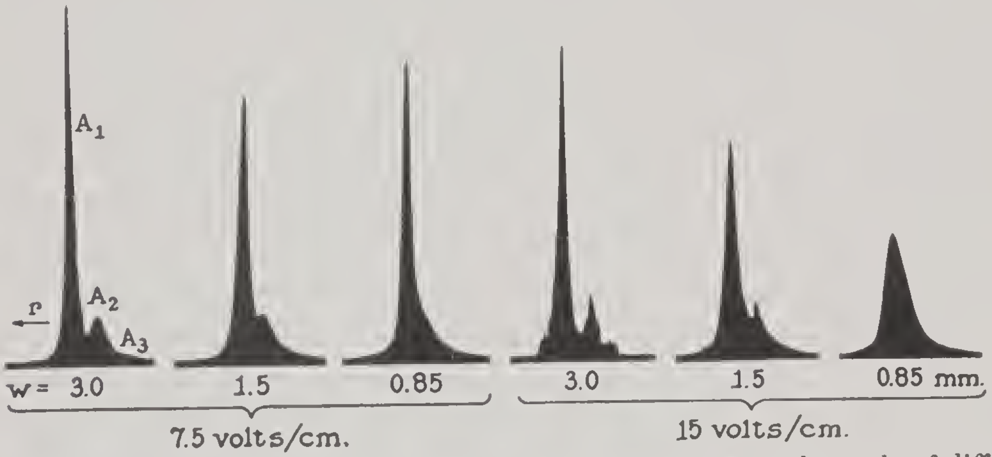


FIG. 15.—Patterns of the rising boundaries of ovalbumin in channels of different width, w, and at different field strengths, E.

Effect of Joule heat.—On the other hand, the irregular gradients in the 3 mm channel at 15 v/cm (Fig. 15) are typical of those encountered when excessive field strengths are used in the Tiselius cell and doubtless result from inadequate dissipation of the Joule heat. No such effects were observed in the narrow channels at either field strength, indicating their superiority as regards heat dissipation. In channels much wider than 3 mm, e.g., those used in the so-called separation cells, the dissipation of the Joule heat becomes increasingly difficult. Although electrophoresis is useful for preparative work in milliliter quantities and is invaluable in piloting the various fractionation procedures that are adaptable to large volumes, it does not lend itself readily to direct separations on a gross scale.

The convection resulting from the Joule heat has always been a serious problem in the electrophoresis of proteins, and one of Tiselius' contributions was the minimizing of this by use of a thermostat regulating near the temperature of maximum density, TMD, 4 C in the case of water. In the neighborhood of the TMD the density is practically independent of the temperature, and although the temperature gradients in the conducting solution are much the same as at other thermostat settings they are not accompanied by the differences of density that produce the convective circulation.

The optimum thermostat setting is not 4 C, however, for 2 reasons. First, allowance must be made for the fact that the mean temperature of the conducting solution is above that of the thermostat. Second, and most important, practically all solutes lower the TMD of water. Thus the TMD for 0.1M sodium acetate is 2.85 C and would be still lower for an acetate buffer of this ionic strength. For the solutions most commonly used in electrophoresis the optimum thermostat temperature is near 0 C. A setting of 0.5 C is convenient in that at this temperature there is little tendency for ice to form on the cooling coil and bridge over to the thermostat, thereby transmitting vibrations from the compressor.

The correlation between disturbances due to the Joule heat and the slope of the temperature-density curve for the solution is close. Moore (36) found, for example, that the disturbances at the serum protein boundaries in a urea-rich buffer at 0 C were not unlike those occurring in the urea-free buffer at 17 C where the slope of the temperature-density curve for water was similar to that of the aqueous urea at the lower temperature. The convection caused the pattern of each boundary to split into several sharp spikes whose number and shape changed erratically with time. Similarly, at 0.5 C and with the same current in both experiments, the author has noted greater disturbances in a 1M sodium chloride solution, TMD $\simeq -10$ C, than in a 0.1M solution of the same salt, TMD =

+2.7 C, where 8 times as much heat was developed due to the higher resistance.

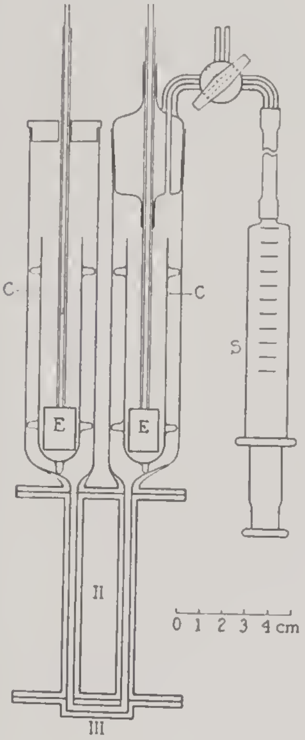
Temperature control.—In most installations the thermostat water is cooled with the aid of a refrigeration unit whose cycling is controlled by a mercury thermoregulator of the sealed type to avoid condensation of moisture at the contact. Although adequate for most purposes, regulation closer than a few hundredths of a degree is not practicable with this method since the compressed gas that is present when the compressor stops continues to pass the expansion valve and lower further the coil temperature. For improved regulation the compressor can run continuously and the excess cooling can be balanced by a thermo-regulator-controlled heater. A preferred alternative, however, is a refrigeration system provided with a solenoid valve between the expansion valve and a "surge" tank (12). The thermoregulator controls the solenoid valve while the pressure in the surge tank controls the cycling of the compressor.

It is essential that both the refrigeration unit, including the cooling coil immersed in the bath liquid, and the motor-driven stirrer be mounted independently of the thermostat and optical system to avoid shaking these components. Excessive vibration of the coil or stirrer can be transmitted through the bath water and should also be avoided. It is preferable to locate the entire equipment on a firm support such as a ground floor or, if an upper floor, near an outside wall.

IX. Electrodes and Electrode Vessels

To keep the products of the electrode reaction from entering the channel, relatively large volumes of buffer are interposed. Although the 700 ml volume of each electrode vessel of Figure 2 is adequate for protracted electrophoresis, in experiments lasting more than a day the neoprene sleeves tend to lose their elasticity at the low temperature. In such cases they may be tied or, preferably, replaced by ball-and-socket joints (57). A more compact electrode vessel (26) that is coming into general use for relatively short experiments, e.g., the analysis of serum, is shown in Figure 16 and simply replaces the top section of the Tiselius cell. The electrode cups *C*, which are removable, serve to keep the 5 ml of 1M sodium chloride solution bathing the electrodes from falling into the channel. Moreover, all rubber connections are eliminated and the cell support is simplified.

Care of electrodes.—The heavy duty silver-silver chloride electrodes, *E* and *E'* of Figure 2, can be "formed" to have a capacity of about 1 amp-hr and can thus carry a 50 ma current for 20 hr. This capacity, combined with mechanical strength, is attained by winding (23) a flat and corrugated strip of 28 gauge sheet silver into a tight spiral (Fig. 17, *a*). The ends of the spirals are anchored with silver screws to a hollow silver core, into which the silver tube *T* is threaded, as shown in cross-section in Figure 17, *b*. The insulating glass tube *S* is then sealed into the core with Pyseal. In the case of the light-duty electrodes, *E* of Figure 16, the Pyseal is eliminated by threading both ends of the silver tube, *T* of Figure 17, *c*, and clamping the glass jacket *S* between neophrene washers *w*. Moreover, these electrodes are made from a $^3/_4$ in. section of $^3/_4$ in. silver rod, the active surface being increased by drilling the holes shown in Figure 17, *d*.



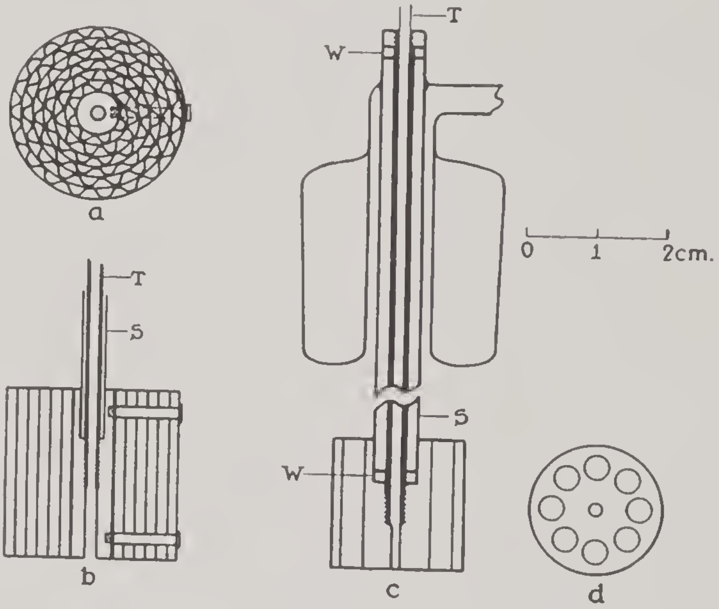


FIG. 16 (*left*).—Compact electrode vessels.
FIG. 17 (*right*).—Construction of silver-silver chloride electrodes.

The reaction taking place at the cathode or negative terminal is

$$\text{AgCl} + \text{electron} \rightarrow \text{Ag} + \text{Cl}^-$$

An adherent, conducting layer of silver chloride must, therefore, be present on this electrode. At the anode the reaction is the reverse, i.e.,

$$\text{Ag} + \text{Cl}^- \rightarrow \text{AgCl} + \text{electron}$$

Since the silver chloride has a larger volume than the silver from which it is formed, the compact structure of the original metal does not favor a high capacity. Before the electrodes are placed in service they should, therefore, be "formed" as follows.

New electrodes may be cleaned and very lightly etched by momentary immersion in dilute nitric acid. After rinsing they are then made the anode and cathode, respectively, in an approximately 1M solution of hydrochloric acid and a current of about 20 ma passed. Hydrogen gas is liberated at the cathode and an adherent brownish-purple deposit of silver chloride forms at the anode. After a time gas, mostly chlorine, begins to be liberated at the anode also. When this occurs the connections are reversed and silver chloride is deposited on what was originally the cathode until it in turn begins to gas. The connections are again reversed and another cycle is begun. This is repeated until the desired capacity is attained. It will be observed that with each cycle more silver chloride is formed at the anode before gas evolution begins here than during the previous cycle. Moreover, as the deposits of "available" porous silver on the one electrode and of silver chloride on the other are thus built up, heavier currents can be carried without loss of active material. The currents should never be of such magnitude, however, as to produce blisters.

If not abused in service, properly formed electrodes will last almost indefinitely. Although procedures differ in the various laboratories, we use one electrode as cathode, say, until the silver chloride is nearly exhausted and then restore the deposit by electrolysis, as in the initial forming of the electrodes, outside of the moving boundary cell. When not in use the electrodes are immersed in distilled water to facilitate the dislodgment of air bubbles that may be trapped on transferring to the buffer solution in the cell.

X. Preparative and Countercurrent Electrophoresis

Many naturally occurring protein mixtures possess 1 or more activities of biologic interest and it is frequently desirable to identify the component with which the activity is associated. The use of electrophoresis for this purpose is now a routine procedure that may be described with the aid of Figure 18. Here the vertical, diagonal and horizontal cross-hatching represents the proteins A, B and C, respectively, whose mobilities in a given buffer are $u_{\mathrm{A}} > u_{\mathrm{B}} > u_{\mathrm{C}} > 0$. The initial boundaries are shown in Figure 18, *a*. On passage of a current in the direction indicated, 3 boundaries will separate and rise (Fig. 18, *b*) to the levels h_{A}, h_{B} and h_{C} in the cathode side while the same number will separate and descend in the anode side. Pure A will then be present between the 2 leading boundaries in the left-band channel and pure C between the 2 slowest boundaries on the anode side.

Recovery of samples.—One procedure for the recovery of a separated fraction for assay follows. If the left-hand channel is open to the atmosphere, a thin-walled glass capillary, attached to the empty syringe of the compensator with small bore rubber tubing, is lowered until its tip is just above the boundary at h_B. With the aid of the compensator the solution of pure A is drawn slowly into the syringe until the boundary at h_A falls to the level of the tip. Similarly, a mixture of A and B would be obtained by inserting the tip to the level h_C and withdrawing the solution between this boundary and the one at h_B. Although suitable for injection, the electrolytic compensator described by Johnson and Shooter (17) could not be used for withdrawing solution.

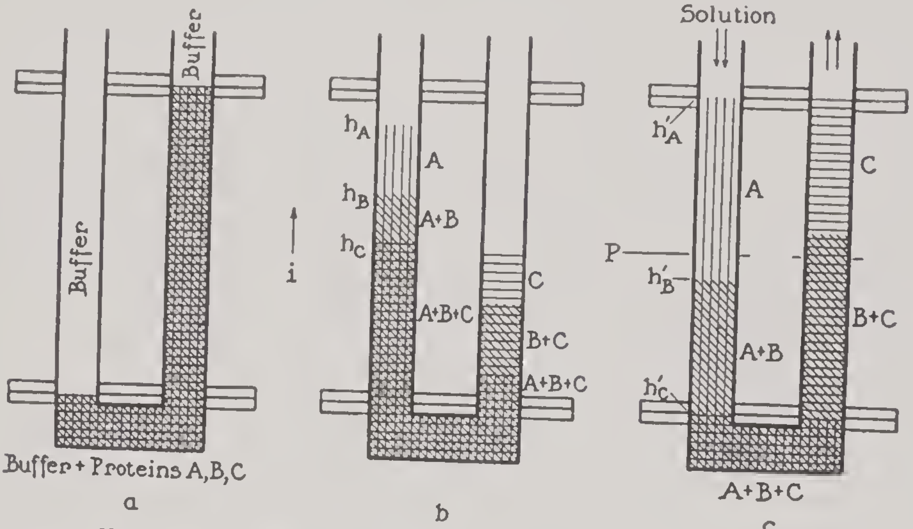


FIG. 18.—Ideal electrophoresis of a protein mixture.

In the case of the solution of pure C in the closed side of the channel, the contents of this side must be immobilized before access is obtained by removal of the stopper *p* of Figure 2. This is done by displacing the bottom section of the cell to one side.

As is evident from Figure 18, *b*, the component B, of intermediate mobility, is still mixed with A in the one side of the channel and with C in the other side. Either of these solutions can, of course, be recovered and used for the separation of B in a 2d experiment. Small quantities of this component can, however, be obtained somewhat more readily as follows (53). After removal of the layer of pure A, the upper boundary in the left-hand side is then between the buffer and a mixture of A + B. If, now, current is passed in a direction opposite to that used in the original separation, B will lag behind A as the species descend and some separation will occur before the boundary due to A enters the bottom section. Similarly, small quantities of B could be separated from C in the right-hand channel.

Countercurrent electrophoresis.—It is also evident from Figure 18, *b*, that if electrophoresis is continued as indicated the boundaries will migrate into the top and bottom sections, respectively, before any large proportions of A and C have been separated. Movement of a boundary into the bottom section always results in convection and must be avoided. If, however, the slowest boundary is given an apparent velocity of zero, as is indicated in Figure 18, *c*, while the leading boundary moves through the height of the channel, much larger proportions of A and C could be separated. Actually a boundary can be given any apparent velocity desired by injection, or withdrawal, at the proper rate, of buffer into the closed side of the apparatus with the aid of the compensator. The movement of the boundaries against a flow of solution in the opposite direction, whereby they are kept in the channel of the cell, is called countercurrent electrophoresis. It has the effect of extending indefinitely the height of the channel and is used not only in preparative work but also in the resolution of protein ions of slightly differing mobilities.

In some instances an early form of the Tiselius cell (Fig. 1, *c*), in which the center section is replaced by 2 identical short ones of the same total height, may be used to advantage in preparative work. Thus if the countercurrent electrophoresis indicated in Figure 18, *c*, were carried out in this cell, the plane of separation between the 2 sections would be at *P* and shifting the top center section would isolate a solution of pure A in the left-hand leg from which it could be readily removed on dismantling the cell.

Limitations.—A comparison of actual patterns of protein mixtures with the diagrams of Figure 18 will emphasize the fact that the system represented here has been idealized. Thus real boundaries spread as they move and the patterns from the 2 sides of the channel are not mirror images of each other. In countercurrent electrophoresis the most serious difficulty, however, is the presence of the concentration boundaries, the so-called δ and ϵ effects, that remain, on passage of the current, essentially stationary at the levels of the original boundaries. With a counterflow the ϵ boundary is carried into the top section while the movement of the δ boundary into the bottom section initiates convection that should be avoided. Since these boundaries arise from the inequality of the Kohlrausch function for the protein and buffer solutions, they may be eliminated by proper dilution (28) of the solutions, protein solution following dialysis. If any appreciable quantity of material is to be prepared by repeated countercurrent electrophoresis, this procedure will save time by affording the optimum yield from each batch.

XI. Micro-Electrophoresis

Commercial equipment.—Although the procedures described here have been restricted to those for the Tiselius cell, it is clear that similar ones would be applicable to the micro- and semimicrocells. Moreover, an apparatus accommodating the standard cell could be adapted for use with the smaller ones. In Table 3 some of the available instruments are listed, together with the optical systems employed, the dimensions of the cell channel and the volume of solution required. The value of h given in this table is the clear height of the channel. It will be noted that in the semimicrocells of the Aminco and Perkin-Elmer instruments and the microcells of the Antweiler and Kern apparatus, at least 2 of the channel dimensions of the standard cell have been reduced in order to decrease the volume of solution required. The relationship of the width and depth of the channel to the electro-osmosis and

TABLE 3.—COMMERCIAL ELECTROPHORESIS EQUIPMENT

INSTRUMENT	OPTICAL SYSTEM	CHANNEL DIMENSIONS, MM			VOL, ML	MFGR OR AGENT
		w	a	h		
Aminco Standard	Schlieren cylinder	3	25*	76	9	1
Aminco Portable	Schlieren cylinder	3	25*	57	6	
Antweiler	Jamin interferometer	1	5	12	0.2	2
Hilger	Schlieren cylinder	3	25	86	10	3
Kern	Jamin interferometer	1.5	5†	30	0.4	4
Klett	Schlieren band, scanning and cylinder	3	25	86	10	5
LKB	Schlieren cylinder	3	25	86	10	6
Pearson	Schlieren cylinder	3	25	86	10	7
Perkin-Elmer	Schlieren band, scanning and cylinder	2	15	45	2	8
Strubin	Schlieren cylinder	3	25	86	10	9

* Semimicrocells also available with a depth of 15 mm.

† Optical sensitivity corresponds to a 10 mm depth since the light is reflected back through the channel.

1. American Instrument Co., Silver Spring, Md.
2. Shandon Scientific Co., 6 Cromwell Place, London, S.W. 7, England.
3. Jarrell-Ash Co., 165 Newbury St., Boston 16, Mass.
4. Kern Co., 5 Beekman St., New York 7, N.Y.
5. Klett Mfg. Co., 179 East 87th St., New York 28, N.Y.
6. LKB Produkter, Box 12035, Stockholm 12, Sweden.
7. Frank Pearson Associates, now associated with Specialized Instruments Corp., Belmont, Calif.
8. Perkin-Elmer Co., Norwalk, Conn.
9. Strubin and Co., Basle, Switzerland.

optical sensitivity, respectively, has already been discussed (p. 93 and equation (1)). The height, on the other hand, is an important factor in determining the resolving power of a given cell. Thus the height required for the resolution of the components of a 4× diluted human serum may be inferred from the patterns of Figure 19 that were recorded, during electrophoresis in 0.1M sodium diethylbarbiturate of pH 8.6 at 4.42 v/cm, at the intervals given in column 2 of Table 4. To conserve space, only the patterns of the rising boundaries are given and only the final one, *g*, contains the complete albumin peak. In column 3 of Table 4 are given the displacements of this boundary in the patterns of Figure 19, whereas the values of column 4 are the minimum heights that include all of the refractive index gradients.

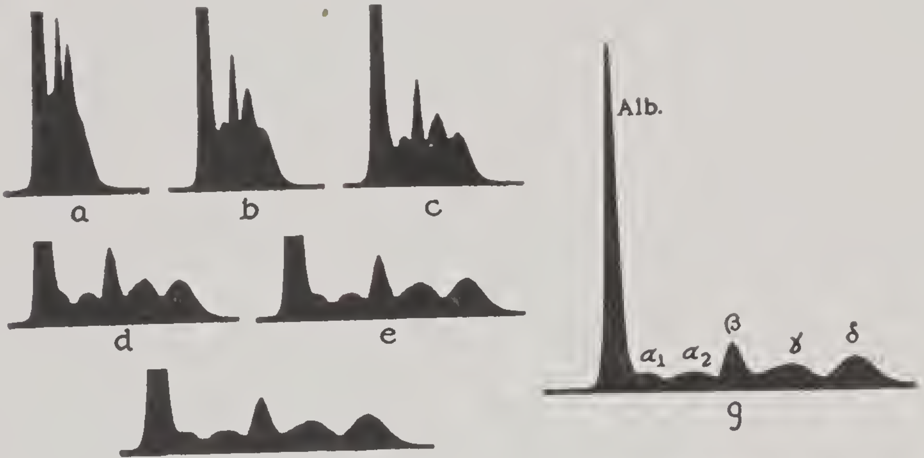


FIG. 19.—Resolution of the serum proteins as a function of the height.

Resolving power.—If by resolution is meant the appearance of a minimum in the gradient curve, i.e., a point of inflection in the curve of refractive index versus height, then only the α_2- and β-globulin boundaries are resolved in the patterns *a* and *b* of Figure 19. In *c* a minimum between the γ-globulin and δ boundary appears, thus permitting the evaluation of the concentration of this component. Separation of α_1-globulin from albumin is the last to occur and, although evident in *c*, is not well defined until the final pattern, *g*.

Also included in Table 4 are the areas, in arbitrary units, of the resolved peaks, from which it is clear that, in general, correct values are not obtained immediately on resolution. This is due to the fact that division of the pattern, as in Figure 4, *d*, causes a diffuse boundary to contribute more to the area of an adjacent sharp one when first resolved than after further separation.

Choice of cell.—If the quantity of material available is small and if the electrophoretic pattern must be obtained quickly, use of the small cells is indicated. To retain the stabilizing effect of the vertical density gradients at the boundaries, it is probably better to examine a limited quantity of material in a small cell at a moderate concentration than to dilute it to the volume required for the standard one. However, resolution in the short cells is inferior to that in the tall ones. Even when allowance is made for the diffusion that occurred on shifting the initial boundary in obtaining the patterns of Figure 19, and for the rather low potential gradient employed, it is probable that in a channel much shorter than 50 mm the α_1 content would be uncertain and in one less than 20–30 mm high the division of the area due to the unresolved γ-δ boundary would be difficult.

TABLE 4.—DEPENDENCE OF ANALYSIS OF SERUM UPON RESOLUTION OF BOUNDARIES

PATTERN	Δt SEC	Δh_{ALB} CM	$h_B - h_P$ CM	ALB	AREA IN PLANIMETER UNITS				
					α_1	α_2	β	γ	δ
a	2,650	0.72	2.0			180	630		
b	3,850	1.08	2.3			239	555		
c	5,350	1.56	2.9			240	519	582	471
d	8,850	2.57	4.1			285	506	523	594
e	11,350	3.30	5.1		119	284	504	546	635
f	13,850	4.04	5.7		172	285	471	497	619
g	16,250	4.73	6.6	2,600	188	277	462	517	635
Apparent composition				64.3	4.6	6.8	11.4	12.8%	

XII. Conclusion

Although more than a decade has elapsed since Tiselius adapted the moving boundary method to the electrophoresis of proteins, neither the apparatus nor the technique has crystallized into a rigid form. This is doubtless indicative of the vitality of the method and of the ever-increasing field for its application. Although the procedures suggested in this chapter are ones that experience has shown are capable of yielding precise results, it will be surprising, indeed, if they do not undergo further refinement and change. The emphasis has been, however, on technique rather than application. In an effort to compensate for this, in part at least, a number of review papers containing extensive references to the literature have been included in the bibliography and are indicated by asterisks. Especial attention is called to Svensson's thesis (48).

NOTE.—This section was reviewed by Duncan A. MacInnes, Gertrude E. Perlmann, Theodore Shedlovsky and Lena A. Lewis.

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ULTRACENTRIFUGATION

EDWARD G. PICKELS, *Specialized Instruments Corporation, Belmont, Calif.*

I. Nature of Sedimentation in Centrifugal Fields

In a gravitational field of force, a particle suspended in a liquid medium of lower specific gravity tends to migrate through the fluid in a downward direction. If the density of the particle is less than that of the medium, the movement is, of course, upward. Assuming the absence of convective disturbances and electrical forces, the rate of migration is governed by the size, shape and density of the particle, density and viscosity of the suspending medium and intensity of the gravitational field. In a dilute suspension of identical particles, all rates will be equal and, for the case of downward sedimentation, those particles originally at the surface form a well defined, moving boundary which demarcates the supernatant fluid from the sedimenting phase. Until the boundary reaches the bottom of the vessel, particles continue to collect there at a uniform rate and the concentration of suspended particles in the "plateau" region between the boundary and the bottom remains constant. Two groups of particles of 2 different sedimentation rates in a binary mixture produce 2 boundaries which become further separated with time, the concentration of each component being indicated by the net change in concentration across its respective boundary. Particles of graded size and sedimentation rate exhibit a blurred boundary which spreads progressively as sedimentation proceeds.

Though well defined, the boundary representing a monodisperse system is not infinitely sharp because of the thermal agitation or brownian movement of the particles. In general, the smaller the particles, the slower the sedimentation rate and the higher the rate at which the boundary diffuses once it is formed. However, a plateau region of uniform concentration continues to exist for a time between the diffuse sedimenting boundary and the lower levels of the fluid which are subject to some backward diffusion of particles which are increasing in concentration at the bottom of the vessel. Sedimentation rates are still measurable, since it is known from the nature of the diffusion process that the position which the boundary would have had in the absence of diffusion is simply the level at which the concentration is one-half that in the plateau region (35).

To produce measurable sedimentation with particles, e.g., protein molecules, in the size range considerably below 1 μ within a reasonable time and without excessive diffusion at the boundary, one must use considerably higher fields of force than afforded by gravity alone. Even though fields in excess of 250,000 times gravity are now routinely used for this purpose, precise measurements of sedimentation rate are still limited to particles having molecular weights above about 10,000, because of the distorting effects of diffusion on the plateau region and on the boundary before it has cleared the meniscus. However, preliminary studies performed with boundaries beyond the meniscus indicate the feasibility of good measurements with molecular weights down to at least 1,000. In pauci-disperse preparations, boundary differentiation is limited to molecular weights of 10,000 or more, depending on relative sedimentation rates, etc.

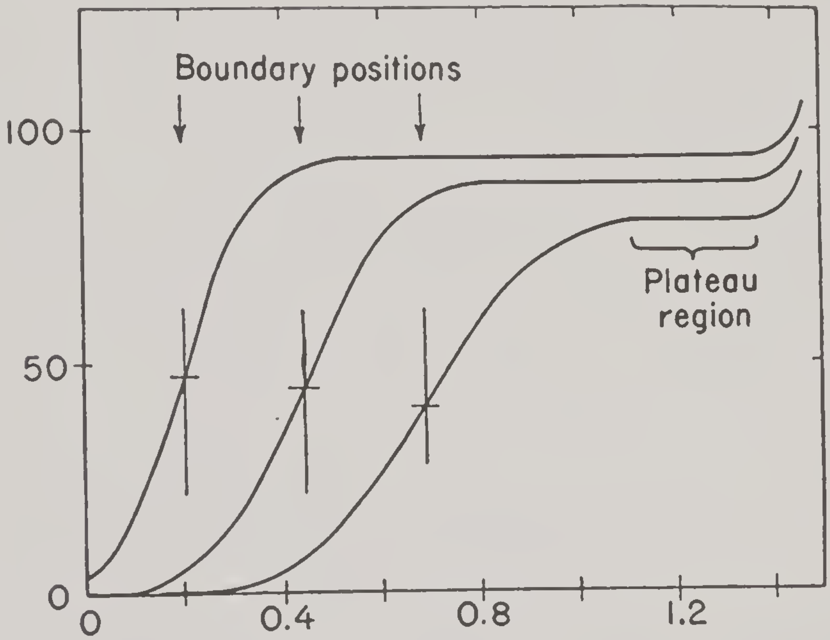


FIG. 1.—Concentration distribution of small "globular" protein (s_{20w} = 2S; $D_{20w} = 10 \times 10^{-7}$; molecular weight = 18,000) at 80 min intervals during ultracentrifugation under average force of 300,000 times gravity. Meniscus distance from axis, 5.8 cm. *Ordinate:* % of original concentration; *abscissa:* distance from meniscus, in cm.

Through the pioneering work of Svedberg and his collaborators (35) since 1923 it has been demonstrated that high centrifugal forces can be utilized in a practical manner for producing precisely measurable sedimentation of molecular particles when certain conditions are met. An essential requirement is that the containing vessel or cell be shaped appropriately for use in a radially directed centrifugal field of force. It is thus made sector-shaped, having 2 flat walls which, if extended, would intersect along the axis of rotation, while the other 2 side walls are perpendicular to the axis. Thus, particles originally close to any of these walls continue during sedimentation to pursue an average course parallel to the wall, so that there is no sedimentation against or away from the walls to introduce convective disturbances.

As illustrated in Figure 1, there is then 1 primary difference between sedimentation in centrifugal and gravitational fields. Since, during centrifugal sedimentation, the particles on the average follow diverging radial paths and since their rate of movement increases with the distance from the axis of rotation, the concentration in the plateau region steadily decreases. Nevertheless, the true boundary position at any time is still very nearly the level at which the concentration is half that of the plateau region at that time. Also, there is a definite mathematical relationship (see page 119) between the concentration of the plateau region and the displacement of the boundary, so that the initial concentration of any differentiated component can still be determined by applying a correction factor. The curves of Figure 1, taken from an actual sample of a small "globular" protein and illustrating the limitations imposed by diffusion on the so-called "sedimentation velocity method," are also useful guides in visualizing the behavior of other larger, but similarly shaped proteins. It can be shown that to a 1st approximation, protein boundaries sedimented to corresponding positions would require sedimentation times inversely proportional to the two-thirds power of the molecular weight, and the boundary spreading would also be inversely proportional to the two-thirds power of the molecular weight. Thus a boundary of 144,000 molecular weight particles would reach the illustrated positions at 20 min intervals and would exhibit only one-quarter as much spreading. Also, at one-half the speed, or one-quarter the average centrifugal force, the same positions would have been reached at 80 min intervals and the boundary spreading would have been one-half as much as with the smaller protein, since boundary spreading is known to be proportional to the square root of the diffusion time.

The sedimentation behavior of a monodisperse substance is generally expressed in terms of its "sedimentation constant" s, which is the sedimentation rate (in cm/sec) per unit field of force (1 dyne/g of mass). Sedimentation constants are usually given in S or Svedberg units, an S unit being 1×10^{-13} cm/sec/dyne of force on each gram (equivalent to 1×10^{-13} sec). The range of sedimentation constants routinely measured by analytical ultracentrifugation procedures extends from about 2S for small proteins, as illustrated in Figure 1, to about 1,000S for some bacteriophages which are more than 10,000 times as massive. So that sedimentation constants may be more directly compared, they are also customarily reduced to standard conditions with respect to the viscosity and density of the medium, as discussed under mathematical relationships (page 119).

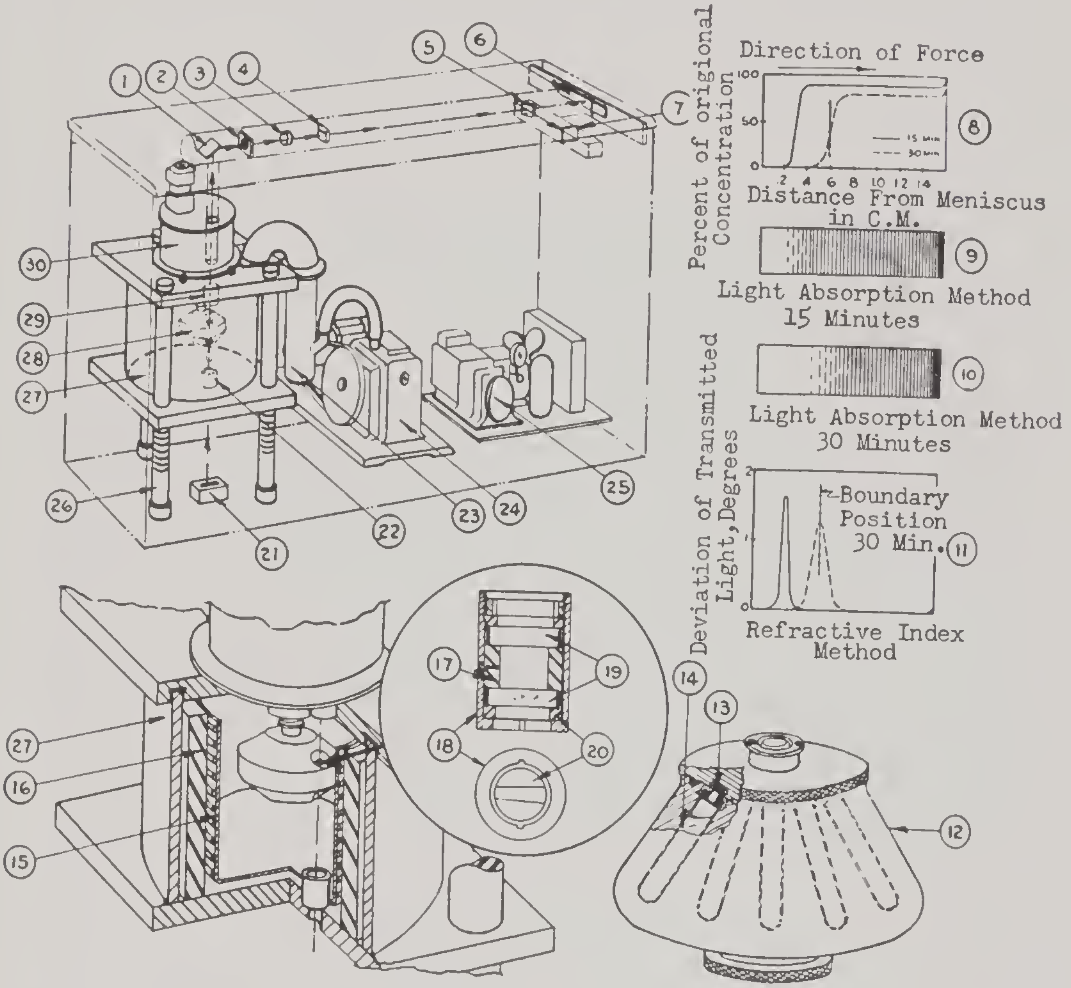
A special case of which very practical use is made is that in which the sedimentation is kept slow enough in comparison to diffusion to prevent formation of any distinguishable boundary. When this situation is continued long enough, with the centrifugal force and temperature held constant, a state of equilibrium is established between the diffusion and sedimentation processes. An analysis of the concentration distribution then permits the determination of molecular weight, in accordance with Svedberg's "equilibrium method" equation (5), given on page 121. Molecular weights of less than 100 can be determined (35). Sedimentation in containers which are not sector-shaped, or under conditions where freedom from convection is not realized, is taken up in section III (p. 126).

The standard reference work for all phases of ultracentrifugation, including theory, method and application, is *The Ultracentrifuge* by Svedberg and Pedersen (35). It also contains an extensive bibliography of publications before 1940. Less extensive but more recent reviews and bibliographies have been published by Nichols and Bailey (19) and Pickels (26).

II. The Analytical Ultracentrifuge

The most precise and routinely used methods for measuring sedimentation are optical. Basic requirements of an optical ultracentrifuge are a rotor capable of withstanding high centrifugal forces, a transparent cell of sufficient resolving power (35), driving means for attaining and maintaining high speeds, a method of avoiding temperature gradients and consequent convective disturbances within the fluid sample, means of measuring and preferably controlling the temperature of the sample, and an optical system capable of recording sedimentation. The early slow-speed machines of Svedberg and Nichols were electrically driven, but in later models, steel rotors with integral, rigid shafts are driven around a horizontal axis, within an atmosphere of low pressure hydrogen, by jets of pressured oil impinging against small turbine blades at the shaft ends (35). Most analytical ultracentrifuges are now made to spin in a vacuum around a vertical axis and are flexibly suspended so as to be self-balancing. The load is carried by compressed air (23), mechanical bearings (27) or, in some cases, by magnetic lift (1). Driving power is furnished by compressed air

(23) or electric motor (27). The speed and resolving power of all modern ultracentrifuges are limited only by the strength of the rotor and its cell. Experience has dictated a distance from the axis of the rotor to the center of the cell of 6.5 cm as being near the optimum for general use (35).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30 Min
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Distance From Meniscus in C.M.
Light Absorption Method 15 Minutes
Light Absorption Method 30 Minutes
Deviation of Transmitted Light, Degrees
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1
0
Boundary Position 30 Min.
Refractive Index Method

FIG. 2.—Schematic drawing of commercially produced, electrically driven ultracentrifuge (27), showing details of transparent cell for fluid sample, light path through revolving cell and method of recording sedimentation. Preparative rotor is also illustrated at right. Complete housed instrument is 74 × 27 × 60 in. For detailed description, see text. (Courtesy, of Specialized Instruments Corp.)

APPARATUS

The design features and working principle of a commercially produced ultracentrifuge (27) are illustrated in Figures 2 and 3. Referring to Figure 2, the sample to be analyzed is sealed within a sector-shaped space provided in a centerpiece, *17*, against which are clamped 2 quartz disks, *19*, with sealing washers of thin plastic sheeting. These, along with a cylindrical housing, *18*, and sector cups, *20*, for the disks, form a transparent cell. Filling is done with a syringe needle through a small hole, which is then sealed with a plastic washer and screw plug. Details of construction have been described elsewhere (24). The depth of the sectoral cavity is 1.5 cm in a radial direction with respect to the rotor, and the thickness between the quartz disks is 1.2 cm. Capacity is approximately 0.8 ml. The cell fits closely into a 1 in. hole provided in the $7^1/_4$ in. rotor, *28*, of aluminum alloy, which is capable of routine operation at 60,000 rpm. The rotor spins within a heavy steel chamber, *27*, which is evacuated to pressures below 1 μ Hg by an oil diffusion pump, *23*, and a backing mechanical vacuum pump, *24*. Added protection against possible rotor explosion is provided by a 2 in. thick steel guard ring, *16*. Opening, closing and automatic locking of the vacuum chamber is accomplished through the rotation of 3 heavy, threaded support rods, *26*.

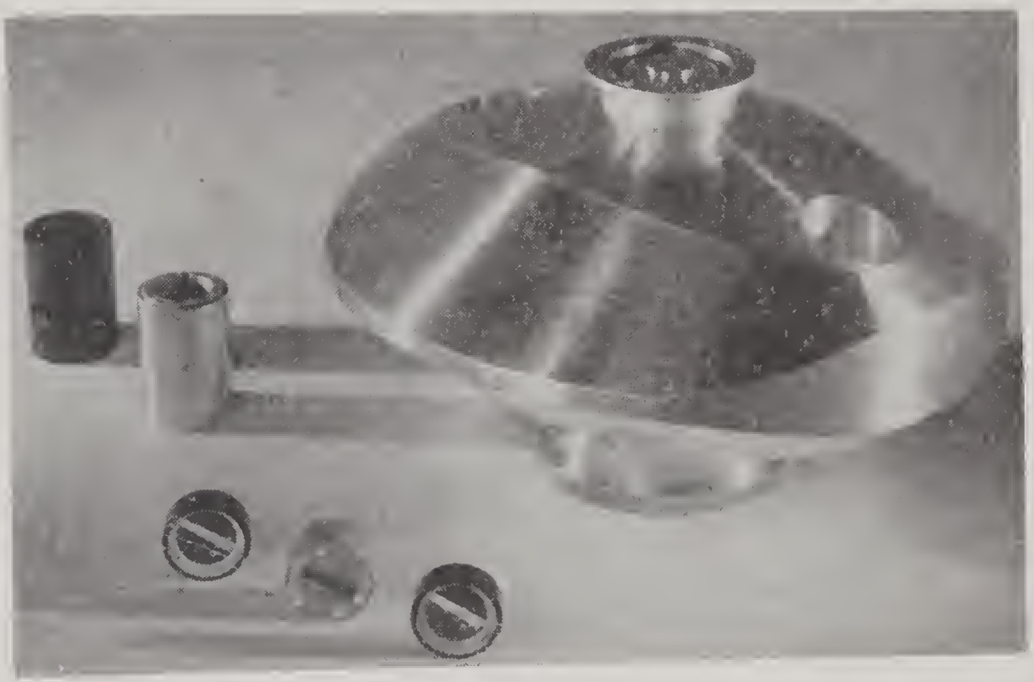


FIG. 3.—Analytical rotor ($7^1/_4$ in. maximum diameter) of aluminum alloy, capable of routine operation at 60,000 rpm (average force on cell, 260,000 times gravity). Cell counterbalance, cell housing, centerpiece and sector cups holding transparent quartz windows are also shown.

When the rotor is operating at full speed with a good vacuum, its rate of temperature rise is only about 1° C/hr when the surroundings are at comparable temperature. Even this rise can be virtually eliminated by adjusting the average temperature of the surroundings about 10–15° below that of the rotor by cooling an enveloping cylinder, *15*, with refrigerant from a compressor unit, *25*. The arrangement is especially useful when one must maintain the rotor at reduced temperatures, as in the case of some unusually labile materials. Most ultracentrifugal runs at high speed are completed within about 3 hr, and to determine rotor temperature for the computation of fluid viscosity, it is quite sufficient to use the mean of values obtained (to within about 0.2° C) with a contact thermocouple applied to the rotor immediately before and after the run. However, a 2d thermocouple element is fixed in position near the coupling device which attaches to the top of the rotor, and its meter indicates approximate rotor temperature continuously during operation. At full speed, after equilibrium is established within about 20 min, the temperature of the fixed thermocouple stays about 2° higher than rotor temperature by virtue of the frictional resistance of the few remaining air molecules. Once the exact difference for a given set of conditions is determined by measurements with the contact thermocouple, the fixed thermocouple readings may be used in corrected form as indicating rotor temperature. The arrangement is especially useful for long runs. Waugh (private communication) has pointed out that theoretically a slight lowering of rotor temperature, proportional to centrifugal force, should occur during acceleration because of the elastic extension of the metal. For 60,000 rpm, the theoretical correction of measurements made on the stationary rotor is about −0.7° C. Temperature changes of this magnitude have been detected by Waugh and by us with "radiation" thermocouples situated on the axis of rotation below the rotor, but our own experiments indicate that the temperature reading is influenced to some extent by the kinetic reaction of the residual gas molecules thrown from the rotor.

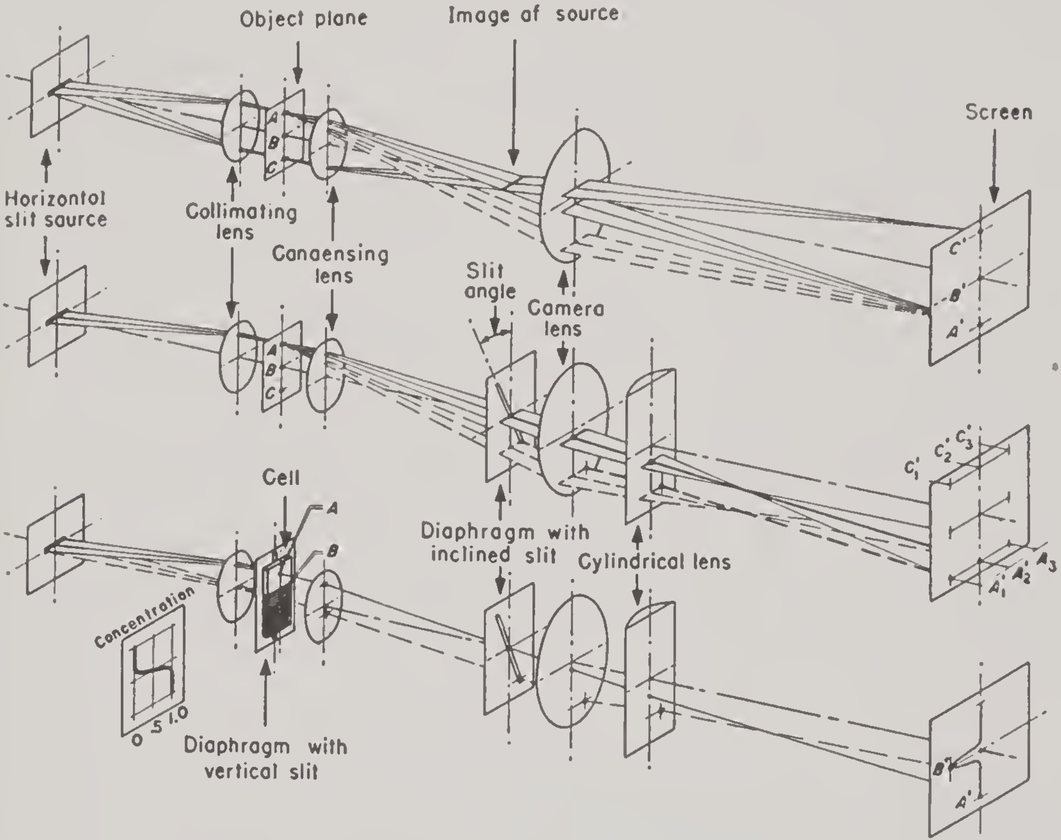
Self-balancing and thermal isolation of the rotor is afforded by a $^1/_{10}$ in. flexible steel shaft which passes through a sealing gland at the top of the vacuum chamber and forms the only connection between the rotor and the electrically operated driving mechanism, *30*, situated above the chamber. Details of the driving mechanism and speed control used in conjunction with it have been described (27). The rotor may be accelerated to 60,000 rpm in 7 min and can be decelerated to rest in about the same length of time. Speed control is provided with 30 different speed settings ranging from 12,000 to 74,000 rpm and with a special low speed attachment, all these speeds can be reduced by a factor of 3. The control works on the principle of matching a reduced speed from the drive against a speed obtained through selected gearing from a synchronous motor. The action of a differential gear governs the supply of electric power to the drive. The average rotor speed is then as closely controlled as the frequency of the power supply and can usually be depended on to within approximately 0.1 per cent.

For detection of sedimentation boundaries (*11*, Fig. 2) by the method detailed in section III, light from a slit source, *21*, is paralleled by a collimating lens, *22*, and directed through the cell within the spinning rotor, *28*. In the region of a sedimenting boundary, the light rays suffer a deviation which the system is designed to measure. The paralleled light is converged by a condensing lens, *29*, to form an image of the slit source at an inclined slit, *2*, after reflection from a mirror, *1*. The light continues through a camera lens, *3*, and a cylindrical lens, *4*, to give on the photographic plate, *6*, and on a viewing screen, *7*, following reflection from a partial mirror *5*, a pattern, *11*, from which the concentration distribution, *8*, of the material within the cell can be determined. Sedimenting boundaries, *9* and *10*, may also be detected through the absorption by the sedimentating material of specific wavelengths of light according to the method of Svedberg (35).

Preparative rotors, *12*, with inclined tube holes of considerable capacity may also be spun in the same machine. The filled plastic or metal tubes, *14*, can be individually sealed, *13*, before being placed in the rotor.

OPTICAL METHODS OF RECORDING SEDIMENTATION

Cylindrical lens procedure.—The optical method most widely used with the ultracentrifuge is that attributed primarily to Philpot and modified by Svensson, who discussed the method in great detail in his thesis (36). A schematic representation of the optical principles on which the method is based is given in Figure 4. For a more complete description, see reference (26). In the simplest arrangement, all elements of the system are centered on an optical axis and are disposed along it so as to meet the following requirements: light rays from a common point in the slit source are parallel after passing through the collimating lens; undisturbed rays (solid lines) are focused by the condensing lens to form an image of the slit source in front of a camera lens as shown; with the cylindrical lens removed from the system (as in the 1st arrangement) light through a pinhole in the object plane is focused by the camera lens to form a sharp image of the pinhole on the screen, regardless of any deflection (broken lines) or scattering of the light artificially introduced at the pinhole. To complete the system (2d arrangement in Fig. 4), a diaphragm with an inclined slit is interposed at the position of the slit image, and a cylindrical lens with axis vertical is so placed that a diffusely illuminated pinhole in the plane of the inclined slit would be focused as a sharp vertical line of light on the screen. It will be observed that if the diaphragm with the inclined slit is now removed, light coming through a pinhole in the object diaphragm is focused on the screen as a horizontal line of light, and its position is the same regardless of any vertical deviation (dotted lines) imposed on the light rays at the pinhole. With the inclined slit diaphragm in place and interrupting all except 1 of the rays within a light "sheet" (rays in single plane) emanating from the pinhole, only a short element of this horizontal line of light remains on the screen. This spot of light is displaced sideward if the inclined slit is moved laterally or if the light sheet is deflected a different amount at the pinhole so as to change the vertical level at which it reaches the inclined slit diaphragm.


Object plane
Image of source
Screen
Horizontal slit source
Collimating lens
Condensing lens
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Camera lens
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C_2'
C_3'
A_1'
A_2'
A_3
Cell
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B
Diaphragm with inclined slit
Cylindrical lens
Concentration
0 .5 1.0
Diaphragm with vertical slit
B'
A'

FIG. 4.—Perspective schematic drawing illustrating working principle of the Philpot-Svensson optical system widely used for ultracentrifugal analysis.

In essence, the system is able to convert a vertical deviation of light at the object plane into a horizontal displacement of a light point on a screen or photographic plate without altering the point's vertical height, which itself corresponds to a certain level in the object plane. Study of the focusing arrangement shows also that the same effect (sharp light point) would be produced on the screen even if a horizontal slit were substituted for the pinhole in the object plane, except that the light point would be brighter on the screen. In recording sedimentation boundaries, as illustrated for an ultracentrifuge with horizontal rotational axis in the 3d arrangement of Figure 4, a diaphragm with a slit a few millimeters wide is used near the cell to avoid lack of definition which would otherwise be caused by curvature of the path followed by any element of the fluid around the axis of rotation. Fortunately, we are concerned only with the redistribution of material in a radial direction, and hence no stroboscopic arrangement to arrest the rotational motion of the rotor optically is necessary. When the cell is in the object plane with the middle of a sedimentation boundary at B, the light rays passing through the solution at B are deviated the greatest amount since this is the region of greatest concentration gradient, and hence of greatest prismatic action. Rays through the supernatant fluid and through the plateau region are undeviated, while those through other portions of the boundary are deviated according to the respective refractive index gradients. A curved line of light of the general character illustrated is thus obtained on the screen, the point of greatest lateral deviation, B', representing the middle of the boundary.

In the simple examples illustrated (see Fig. 2 also), the curve is essentially a graphic plot of the refractive index gradient (directly proportional to concentration gradient) for all levels within the fluid column. If the effect is integrated from the region just above the boundary to the plateau region just below it, one obtains the total refractive index change associated with the boundary and hence the concentration of the material which constitutes the boundary, assuming that the refractive properties of the material are known. In other words, the concentration of any resolved component may be determined by measuring the area under the respective peak (down to the base line corresponding to zero refractive gradient) of the refractive index gradient curve, and applying a formula involving the physical constants of the system (p. 119). For measurement of sedimentation rates, the system is usually ideal, since the boundary position for a dilute homogeneous material is given by the tip of the peak.

In actual practice, the simple method outlined for determining the concentration of a component would apply accurately only at very low speeds of rotation. In high fields of force there are extraneous deviations of light due primarily to the hydrostatic compression gradient set up in the fluid and secondarily to the minute displacement, distortion and compression of the quartz windows within the cell. The partial sedimentation of salts or other low molecular weight materials which diffuse rapidly enough to produce gradients throughout the fluid also has an effect. For approximate area measurements, the displaced base line pertinent to a particular boundary can usually be interpolated from the flat or gently curving sections of the pattern on either side of the boundary. But for the most accurate measurement, one must obtain a "reference base line" by making a duplicate run with the suspending medium alone, the photograph being taken at the same stage of the run. The difference in the patterns, obtained by matching the undeviated references, represents the effect due to the particular component or components not included in the suspending medium. In comparing photographs (especially when only 1 hole is used in the cell counterbalance) and in accurately measuring boundary positions, it is desirable to have the meniscus and the radial level in the rotor corresponding to the counterbalance hole well defined across the whole photograph. This is not accomplished when an inclined slit (Fig. 4) is used.

Instead of having a light pattern on a dark background, it is best to use a dark pattern on a light background. Hence, an inclined opaque bar about $^1/_{16}$ in. wide or a straight wire about 0.010 in. in diameter is usually employed instead of an inclined slit. In measuring areas from photographs obtained with the wire, one should follow the middle of the pattern, which has a minimal apparent width and sharpness regardless of wire size because of optical diffraction. Also by reason of diffraction, the apparent width of the pattern (measured parallel to the meniscus line in the photograph) obtained with the bar changes with slope of the curve and with optical density of the photographic negative. To avoid errors which might amount to 15–20 per cent in the case of very light photographs, one should trace both edges of the pattern and use the mean. In general, the wire is preferred for simple patterns because of easier tracing, but in the case of sharp multiple boundaries close together, the bar pattern usually affords better resolution.

I have also been able to demonstrate that patterns for 2 separate cells run simultaneously in the same rotor can be recorded in usable form in a single photograph. The usual counterbalance is replaced by a "prismatic" cell which is the same as the standard cell except that the outside surface of the upper quartz disk and the corresponding surface of the sector cup are sloped (but still flat) at about 1° to a line which perpendicularly intersects the axis of rotation and the axis of the cell. This gives a separation of the 2 patterns. Some loss in optical contrast and a limitation on the amount of light deviation which can be recorded must be tolerated. However, a tremendous saving in time is afforded when a large number of samples must be run, and routine use has been made of the scheme with good success in certain studies (9). Although light through the air

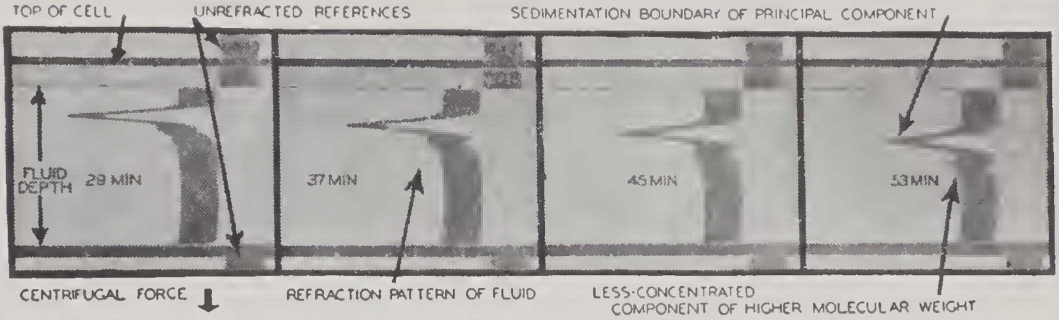
TOP OF CELL
UNREFRACTED REFERENCES
SEDIMENTATION BOUNDARY OF PRINCIPAL COMPONENT
FLUID DEPTH
29 MIN
37 MIN
45 MIN
53 MIN
CENTRIFUGAL FORCE
REFRACTION PATTERN OF FLUID
LESS-CONCENTRATED COMPONENT OF HIGHER MOLECULAR WEIGHT

FIG. 5.—Serial photographs illustrating use of the Philpot-Svensson optical system, with inclined bar instead of slit, to photograph sedimentation in a 1 per cent solution (medium of 0.2M NaCl) of gamma globulin ($s_{20w} = 7.2S$ for main component) at 59,780 rpm. Light to form unrefracted references comes through holes in the cell counterbalance.

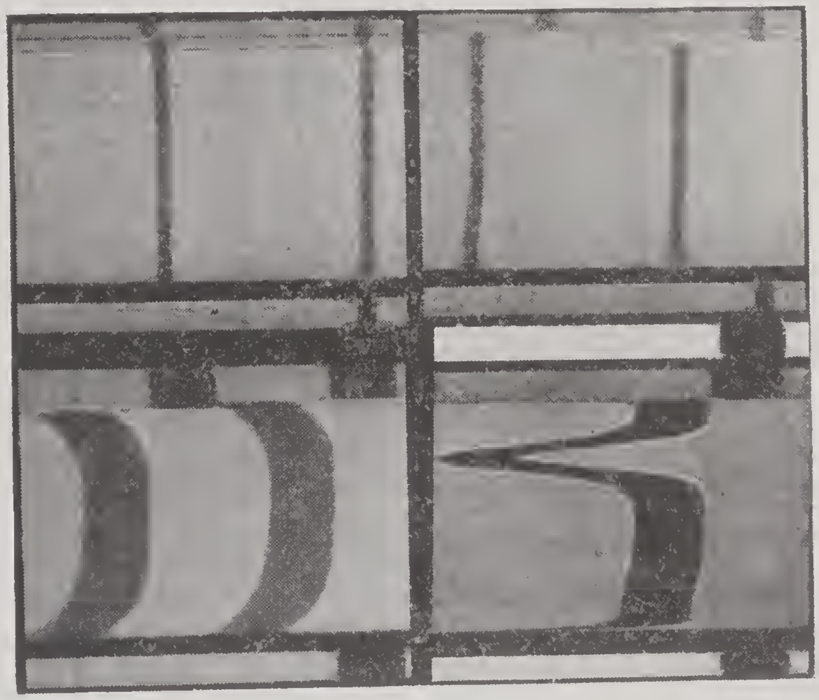

FIG. 6.—Photographs of refractive patterns obtained with an inclined wire (*above*) and an inclined bar (*below*). That at *lower right*, showing 2 references from cell counterbalance, is of serum albumin which is monodisperse except for very small amount of more rapidly settling material. Other photographs show patterns obtained when both a normal and a "prismatic" cell are run simultaneously, with water at low speed (*upper left*), water at 52,640 rpm (*upper right*), and 1.5M NaCl after 15 min at same speed (*lower left*).

bubble above the fluid can be used as an undeviated reference, a more dependable and better defined one is obtained by drilling a small vertical hole through the edge of the rotor in a position at right angles to the 2 cells. The various points discussed above are illustrated in Figures 5 and 6.

Modified scale method.—Another optical method which gives the same sort of information as the one described is the scale method of Lamm, later simplified by Svensson (37). Scale lines are photographed through the cell and the displacements of the line images are measured and graphically plotted to give the refractive index gradient curve. The method is tedious, although it is least susceptible to the small optical errors that might be significant if one wishes to determine refractive index gradients with the highest possible precision. It is most ideally suited for sedimentation equilibrium studies but is hardly justified in the measurement of sedimentation rates.

Absorption method.—Boundaries may also be detected by the absorption method of Svedberg (35), but complete analysis of the photographs is not nearly so direct or absolute in measurement and the method is seldom used except for certain special applications. Ultraviolet light is usually employed, and in the case of nucleoproteins and certain other materials which absorb ultraviolet light very strongly, some advantage is gained in the way of extending measurements to very low concentrations or unusually inhomogeneous material or of differentiating highly absorbing materials in a mixture.

MATHEMATICAL RELATIONSHIPS

The derivations of equations for sedimentation constant, molecular weight, frictional coefficient, and concentration correction factor have been given by Svedberg and Pedersen (35), and these should be consulted for a complete understanding of ultracentrifugal theory. Only the more important of these equations are restated here, along with some less quoted relationships which I have found useful. The movement of a sedimentation boundary in a centrifugal field is described in terms of a sedimentation constant, s

$$s = \frac{(\log x_2 - \log x_1)}{\omega^2(t_2 - t_1)} \qquad (1)$$

where ω is the angular velocity in radians/sec ($=2\pi \times$ rps) and x is the distance in centimeters of the boundary from the axis of rotation at time t. A sedimentation constant is usually expressed in S or Svedberg units and, when not otherwise indicated, it is generally assumed to represent the value obtained by extrapolating experimental results to zero concentration, since sedimentation rate changes with concentration. Also, for purposes of comparison, sedimentation constants are generally reduced to values which theoretically would be obtained if the medium had the viscosity and density of water at 20 C (subscript $20w$)

$$\frac{s_{20w}}{s} = \frac{\eta(1 - V\rho_{20w})}{\eta_{20w}(1 - V\rho)} \tag{2}$$

where η is the viscosity of the medium, ρ is the density of the medium, and V is the partial specific volume of the sedimenting material. This corresponds to the increase in volume when 1 g of dry solute is added to a large volume of the medium and is equivalent to the reciprocal of the density of the material (in the unhydrated, unsolvated state unless otherwise designated). This equation assumes that V would have the same value in the standard medium, and it also assumes, when V is taken for the unsolvated state, that the sedimenting particles are solvated with material of the same density composition as the surrounding medium and to the same extent volumetrically in both mediums. Usually, the experimental medium and hypothetical standard are similar and the densities of solute and medium are not too close together. The equation can then be applied with some assurance. When these conditions are not realized, appreciable error can be introduced unless proper correcting factors are applied (31). V has a value near 0.74 in the case of many proteins.

Molecular weight, M, can be computed from Svedberg's formula, which assumes that the particles are solvated, if at all, with material having the same density as the medium

$$M = \frac{RTs}{D(1 - V\rho)} \tag{3}$$

R is the gas constant (8.313×10^7), T the absolute temperature, and D the diffusion constant which is usually measured in a separate experiment by standard techniques (20). Values of s and D must be for very dilute concentrations (preferably extrapolated to zero concentration) and should ideally be for the same medium at the same temperature. However, an equation analogous to (2) can usually be applied with negligible error to correct s values to the conditions of the diffusion experiment.

The diameter, d, of spherical particles (applies strictly for unsolvated particles unless V is for solvated material) or a minimal possible value for the average diameter of particles of unknown shape is given, in the case of dilute solutions, by

$$d^2 = \frac{18\eta sV}{(1 - V\rho)} \tag{4}$$

For sedimentation equilibrium in a monodisperse system, the Svedberg formula for molecular weight is

$$M = \frac{2RT \cdot \log\left(\frac{C_2}{C_1}\right)}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)} \tag{5}$$

where C_1 and C_2 are concentrations at radial distances, x_1 and x_2. Better suited for use with the refractive index system described is the form

$$M = \frac{2RT \cdot \log\left[x_1\left(\frac{dc}{dx}\right)_2 \Big/ x_2\left(\frac{dc}{dx}\right)_1\right]}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)} \tag{6}$$

In equations (5) and (6), the same assumption is made regarding solvation as in equation (3).

The relation between the concentration of a particular component in its respective plateau region at any time t and the original concentration C_0 is

$$C_t = C_0\left(\frac{x_0}{x_t}\right)^2 \tag{7}$$

where x_0 and x_t are the respective radial distances of the boundary at zero time (meniscus or bottom of cell) and at time t.

From patterns obtained with a Philpot-Svensson optical system using paralleled light through the cell, the concentrations of sedimenting components can be computed by applying the following equation

$$C_0 = \left(\frac{A_t \cdot \tan\theta}{L \cdot m_1 \cdot m_2 \cdot H \cdot \Delta n}\right)\left(\frac{x_t}{x_0}\right)^2 \tag{8}$$

θ is the angle between the undeviated slit image and the inclined wire, bar or slit; L is the distance in centimeters along the optical path from the nodal point of the condensing lens (midplane of lens, approximately) to the inclined wire; m_1 is the magnification of cell height on the photographic plate, and m_2 the magnification of the cylindrical lens system, determined by photographing scale lines which are placed in the same plane as the inclined wire and parallel to it with $\theta = 90°$. H is the thickness of the fluid column along the optical path, in centimeters, and Δn is the specific refractive increment of the particular component (equal to 0.00186 in the case of bovine serum albumin, for example, when yellow light is used and the protein is in a 1 per cent solution of NaCl). A_t is the area in square centimeters associated with the particular component on a photograph taken at the time t. When boundaries are not completely differentiated, reasonably good determinations can still be made if the area is apportioned by drawing a straight line, parallel to the meniscus, from the reference base line to the point of closest approach on the curve of the sedimenting material. A better method is that of drawing a gaussian curve for each component in such a manner that the several curves account for the total concentration.

The Performance Index, P_i, is a rating proposed by the author to be applied to preparative rotors, with inclined tubes or with fluid cavities of any shape, to express approximately their relative performances in accomplishing the complete precipitation of any given material under certain idealized conditions. It depends only on the physical dimensions of the rotor and its speed.

$$P_i = \frac{(\text{rpm})^2}{\log R_2 - \log R_1} \qquad (9)$$

where R_2 and R_1 refer to the maximal and minimal radial distances (from axis of rotation) in centimeters of the contained fluid.

EXPERIMENTAL REQUIREMENTS AND PROCEDURE

Most macromolecules are electrically charged with respect to the suspending medium, and in sedimenting they attempt to drag along through the medium those surrounding ions for which there is an electrical attraction. Sedimentation rates may be reduced by more than 20 per cent in some cases unless this Donnan effect is repressed through the addition of low molecular electrolyte to the medium. For most proteins, the reduction in rate is less than 1 per cent if 0.2 mole of sodium or potassium chloride is added for each 1 per cent of protein (35). In the study of small molecules by the equilibrium method, equations allowing for charge effect must be used (35). Also, it should be emphasized that in citing ultracentrifugal results, one should fully specify the composition of the test sample. Concentration of the solute, pH, temperature, concentration of dissolved salts or other materials and the previous history of the sample may all have some effect on the corrected sedimentation constant or the degree of ultracentrifugal homogeneity.

Sedimentation constants should be measured at several different concentrations and the results extrapolated to zero concentration for assigning values applicable to the equations given earlier. In the case of "globular" proteins, the sedimentation rate is generally decreased in the neighborhood of 8 per cent for each 1 per cent of protein. The effect increases with asymmetry in particle shape. If data are limited to a preparation of fixed composition, e.g., undiluted blood serum, or if for any reason s values have not been extrapolated to zero concentration, then the viscosity of the complete preparation and approximate correction factors for each component, e.g., +8 per cent/1 per cent protein, can be used in applying equations (2) or (4) to obtain fair approximations to true values.

A discussion of the effects of concentration and polydispersity has been presented by Kinell (13). When concentrations are above 1 or 2 per cent boundary sharpening effects may complicate the analysis. Also, with mixtures in which the sedimenting components contribute appreciably to the viscosity of the solution, sedimentation behavior will in general depart from that observed at infinite dilution. Johnston and Ogston (12a) have studied this phenomenon. The influence of hydrostatic compression on density and viscosity may generally be disregarded with aqueous solutions but must be corrected for in the case of organic solvents (26).

Sedimentation rates are best determined by taking photographs at intervals and graphically plotting log x against t. The equivalent starting time ($t = 0$, and radial displacement of meniscus or bottom of cell for x_0) is time at which approximately two thirds of operating speed is reached, assuming nearly uniform acceleration. This point, which can usually be employed with small error despite reflected diffusion while boundary forms, is valuable where boundaries of a mixture do not resolve until late in the run. For example, with the ultracentrifuge illustrated, resolution is barely obtained with equally concentrated "globular" proteins of 18,000 and 40,000 molecular weight. For best results with the optical system illustrated, the illuminating slit width should be limited to about $^1/_{10}$ mm, approximately monochromatic light should be used, and the photographic exposure time should not be more than about 2 per cent of the time required for the boundary to traverse the cell. When it is not practical to determine the diffusion constant in a regular diffusion apparatus, an approximate value can be obtained from the boundary in the ultracentrifuge, especially if measurements are made after the rotor is brought to a very low speed following a rapid migration of the boundary to the middle of the cell at high speed (26). Monodisperse boundaries are sharpened while in motion because of the variation of s with concentration; on the other hand, inhomogeneity in rate during sedimentation may give the appearance of unduly rapid diffusion. The first and last photographs taken at speed should always be compared to make sure that no leakage of fluid has occurred.

Accurate measurement of concentration from refractive patterns requires a precisely shaped cell centerpiece and its proper alinement in the rotor. For the solute run and the reference base line run, all cell parts should be identically positioned and oriented, only the sealing washers should be changed, the cell should be tightened and filled to the same degree and preferably run in the same rotor hole. If photographs show that the cell was not filled identically, meniscus positions should be matched in determining the true base line for the inner half of the cell; cell bottom positions should be matched for the outer half of the cell. Small "spikes" or "raggedness" in the pattern generally denote localized convections which could be, but seldom are, due to leakage or poor vacuum. Their appearance at constant speed may indicate rough, incorrectly shaped or improperly oriented walls in the centerpiece; or they may simply denote local instabilities in the density distribution set up within the fluid by the sedimentation process. Especially when a high concentration of a highly diffusible salt is incorporated in the medium (see Fig. 6), an appreciable redistribution of density is accomplished within a short time. The slight reduction in sedimentation or "flotation" rate (9) of protein particles (in their own plateau region) as they move into the plateau region of the salt where their rate should be uniform can result in a "piling up" which tends to produce a layer of fluid that is more dense than the adjacent layer on which it is supposed to float. A "hump" which always appears in the refractive pattern at the same level of the cell, with solute but not with the medium alone, suggests lack of flatness in sector walls.

In starting a run, it is good practice to take 1 photograph at low speed (about 4,000 rpm) and another as soon as operating speed is reached following rapid acceleration. Any failure of the refractive pattern (excepting regions near meniscus and cell bottom) in the 1st full speed photograph to match the corresponding reference base line from the solvent run is usually caused by the settling of very large particles. Differences in optical density and sharpness of pattern between the low and high speed pictures is substantiating evidence of rapidly settling particles, e.g., denatured protein. Appreciable curvature in the low speed pattern, or failure to match the corresponding pattern obtained with solvent only, indicates an unclean or incompletely dried cell centerpiece, a lack of complete mixing in the sample, or faulty assembly of the cell.

Whereas sedimentation velocity runs never require more than a few hours with aqueous mediums, sedimentation equilibrium runs require considerable time and, except for materials of very low molecular weight, relatively low speed (see (35) for details). For example, a monodisperse "globular" protein of 40,000 molecular weight would require about 4 days of steady running at an appropriate speed of about 11,000 rpm. It will be noted that equation (6) can be applied for a monodisperse protein, even if the refractive increment of the material and the optical constants of the instrument are unknown. If polydisperse materials are to be studied by the equilibrium method, one needs such information and should apply it to other formulas as outlined by Wales *et al.* (39).

The separation cell of Tiselius, Pedersen and Svedberg (35), though not an ideal solution to the problem, does offer some help in correlating sedimentation boundaries with biologic or chemical properties. Two halves of the cell, which can be individually sampled, are separated by a porous partition. Whether or not a boundary has crossed the partition before stopping of the ultracentrifuge can be observed with the optical system.

ACCURACY OF METHOD AND INTERPRETATION OF RESULTS

With careful techniques and a stable material, one can repeat determinations of sedimentation constant to within less than 1 per cent or to within a few hundredths of a Svedberg unit in the case of low *s* values. Kegeles and Gutter (12b) have been able to reproduce values for proteins to within less than 0.01 Svedberg unit by determining boundary positions from the Fresnel diffraction patterns which occur in the photograph at the boundary when an inclined bar is used with the refractive method already described. However, average deviations for values appearing in the literature are sometimes several times as great, probably most often owing to the unstable nature of the material or to small errors in the measurement of rotor temperature. An error of 1° C usually causes an error in the *s* value of more than 2 per cent. With monodisperse systems of large particles, concentrations as low as 0.01 per cent can be easily detected and measured. Ten times as much material may be required for low molecular weight materials. In the measuring of protein concentration, for example, results can be reproduced (at least in the same ultracentrifuge) with a mean deviation of about 0.01–0.03 per cent, depending on the sharpness of the boundary.

As a general rule, when the refractive pattern of a boundary is symmetrical around the point of greatest refractive index gradient, the material constituting the boundary is very nearly homogeneous with respect to sedimentation rate. Of course, comparisons can be made with results from diffusion experiments or between boundaries brought to the same level in the cell at different rotor speeds. Kinell (13) and others (35) have treated the subject of polydisperse materials. It should also be noted that aggregates of 2 or 3 primary particles may produce discrete secondary boundaries. In addition to determining sedimentation rate, molecular weight, homogeneity and concentration, one may also gain some information regarding particle shape if the approximate degree of solvation is known or can be determined, as by the method of Sihtola and Svedberg (32). If, for example (21), a protein of known molecular weight is hydrated with 20 per cent water (not significantly different in density from medium generally), the partial specific volume and the diameter (assuming spherical shape) of the hydrated particles can be computed and applied to equation (4), from which a theoretical s value for spherical particles can be obtained. The ratio of this to the experimentally observed value gives a "frictional ratio" from which an "axial ratio" for the particles can be computed.

REPRESENTATIVE APPLICATIONS

For a partial list of publications dealing with applications of the ultracentrifuge, one may consult bibliographies prepared by Svedberg *et al.* (35) and by others (19, 26). It is impossible to summarize or to do justice in a limited space to the thousands of reports on the ultracentrifugation of materials of many sorts. To give some idea of the versatility of the method as applied to substances of interest in the field of medicine, I have included in the present bibliography some publications selected at random with no attempt to pass on their relative importance. The studies on plasma or serum proteins (2, 3, 18, 21, 34, 35) have been particularly fruitful. It has been well established that these proteins have molecular weights ranging from about 69,000 in the case of albumin to several hundred thousand for some of the globulins, and even to several millions in the case of some respiratory proteins from the invertebrates. Most plasma proteins have been found to have axial ratios ranging from about 1 to 6, but, not too surprisingly, fibrinogen appears to have a ratio of almost 20 (21). Recent work of particular interest is that of Gofman *et al.* (8, 9) on the lipoproteins as related to atherosclerosis. In their work, the lipoprotein fractions are separated from the other serum proteins by a flotation process involving preparative ultracentrifugation after the addition of considerable salt to the serum. In the analytical ultracentrifugation of these fractions, the boundaries also move toward the axis of rotation through the salt medium.

Studies have also been made of whey proteins (5), ovomucoid (6), influenza virus (7), visual purple (10), mitochondria (11), bacteriophage (15), tobacco mosaic virus (16), hormones (17), pepsin-treated globulin (22), hemocyanin as affected by Xrays (29), ferritin (30) and chicken feather keratin (40).

III. The Preparative Ultracentrifuge

Although the ultracentrifuge illustrated in Figure 2 can be used for preparative purposes, simpler machines, such as that illustrated in Figures 7–9 are generally used.

Material to be centrifuged is placed in test-tube–shaped containers of plastic or metal which can be individually sealed with special caps having a rubber washer that expands against the container wall when the cap assembly is tightened (Fig. 7). Weighing of the tubes for balance is not necessary because of the self-balancing features of the machine. Sealed tubes are placed into holes of an aluminum alloy rotor, *4*, with enough water first placed in the hole to fill the space between tube and wall of the hole. The vacuum chamber, *6*, is evacuated to a pressure below 100 μ ordinarily by the mechanical pump, *17*. As in the case of the analytical machine, there are a guard cylinder, *5*, and a liner, *2*, which is refrigerated by a compressor unit. The electric drive, *1*, is situated below the vacuum chamber, and a $^3/_{16}$ in. steel shaft from it is fitted at its upper end with a spud on which the rotor rests. Extra cooling is

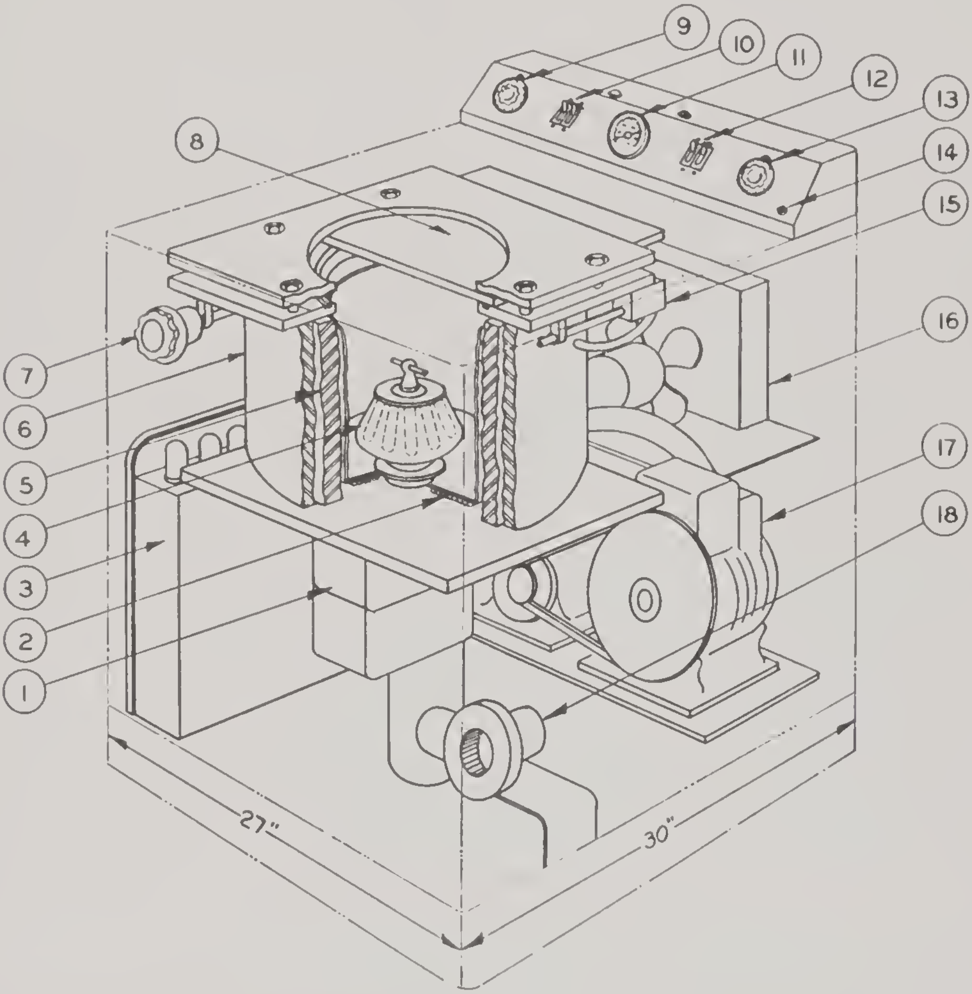

FIG. 7.—Schematic drawing of a commercially produced, electrically driven ultracentrifuge designed for preparative centrifugation of macromolecules. Maximal routine speed is 40,000 rpm. For description, see text. (Courtesy of Specialized Instruments Corp.)

furnished by a blower, *18*. Details of the drive and other parts of the machine have been described (28).

Through a manual control, *7*, the steel lid, *8*, of the vacuum chamber can be drawn into position and allowed to drop onto a large vacuum-sealing gasket. Another control (not shown) governs opening and closure of the air valve, *15*, and starting of the vacuum pump. A time clock, *9*, controls the length of the run, and a speed adjustment, *13*, provides preselection of any speed up to 40,000 rpm. Switches, *10*, *12*, beside the speed indicator, *11*, control the power supply and refrigerator and allow for preselection of braking rate. After the rotor, which carries its own handle (Fig. 8), is in place and the chamber has been evacuated for 1 or 2 min, the machine is started with a push button, *14*, and goes through the complete cycle of operation automatically, controlled by an electronic unit, *3*. A 7 in., 12-tube rotor of 160 ml capacity accelerates smoothly up to the maximum speed of 40,000 rpm (144,000 times gravity) in 4 min, is held at constant speed for the selected time, and is then decelerated in a minimum time of about 4 min to a low speed from which it then very gradually drifts to rest. Temperature

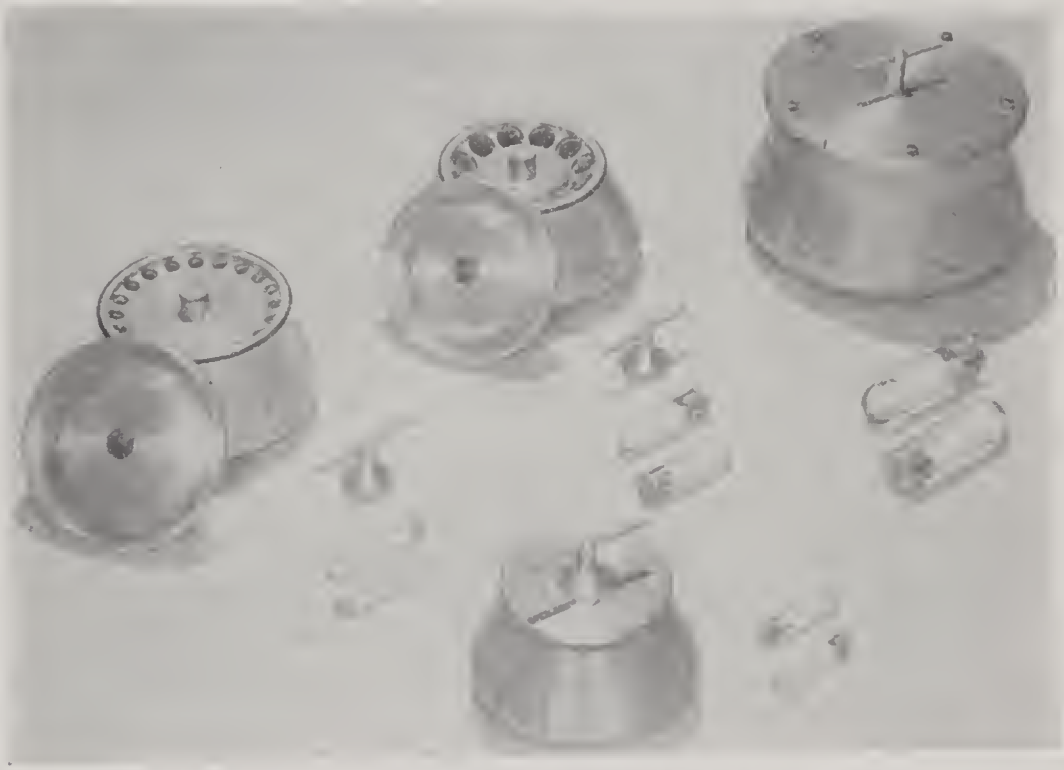

FIG. 8.—Interchangeable rotors of aluminum alloy and flexible plastic tubes used with the preparative ultracentrifuge (Fig. 7). Smallest rotor (7 in. diameter, 160 ml capacity) has routine speed rating of 40,000 rpm; the largest (11 in. diameter, capacity approximately 1 l) is rated at 20,000 rpm. (Courtesy of Specialized Instruments Corp.)

rise, even at top speed and without refrigeration, amounts to only a few degrees centigrade per hour for runs at room temperature. The tubes range in diameter from $^1/_2$ in. (2.5–3.5 in. long) to $1^1/_2$ in. (4–5 in. long). A 1 l rotor with inclined tubes requires about 15 min for acceleration to its limiting routine speed of 20,000 rpm. Tube angles (to axis of rotation) ranging from 14 to 40° are used. Each rotor is provided with a "centrifugal fuse" which, when activated by accidental overspeeding of the rotor appreciably above its rating, will interrupt the circuit supplying power to the drive.

The nature of sedimentation in inclined-tube rotors has been described (25). In general, precipitate is initially collected at the bottom of the tube at a more rapid rate than if the same fluid were contained in a sectoral cell with the inner and outer edges of the fluid at the same radial distances from the axis of rotation. However, assuming the absence of any convective disturbances excepting that caused by the centrifugal concentration of sedimenting particles against the side walls of the tube, the last particles to reach the bottom of the tube require approximately the same total time as if they had moved between the same radial distances in a sectoral cell. Hence the approximate time to produce a nearly complete precipitation of a material of known sedimentation constant can be predicted (if due allowance is made for diffusion, if significant), and equation (9) would appear to afford a fair basis on which to compare various rotor designs.

As a matter of fact, under appropriate conditions sedimentation boundaries can actually be detected and rates measured by examination of tubes with partially precipitated material or by a sampling of the fluid column and a testing of the aliquots for some specific property (25, 7, 4). In such studies, best success is had by using tubes of small diameter ($^1/_2$ in.), an appreciably large tube angle (35–45° preferably), high concentrations of material (1 per cent or more) and a good vacuum. Because of thermally induced convection, results with rotors spinning in the open air are generally poor; in the case of vacuum ultracentrifuges, negligible increase in efficiency is accomplished by the addition of an oil diffusion pump except in cases of unusually difficult separations. The incorporation of a synthetic density gradient of sucrose or other material in the solution as a stabilizing agent is necessary in the case of very low concentrations (25, 7).

Hollowed-out batch-type rotors (Fig. 9) are convenient for handling very large volumes of fluid, especially with materials of relatively high sedimentation rate. The amount of fluid must be such that during operation it almost touches a solid removable core in order to prevent sloshing and the development of excessive unbalance during starting and stopping. Sediment may be resuspended from the grooves at the periphery by turning the rotor with its axis horizontal while stainless steel balls are allowed to roll around inside.

It should be recognized that in large tubes and in single-cavity rotors, a little restirring of sediment from the top, uncompressed layer of the sediment might be expected during deceleration, since the fluid tends to keep up the rotation. Also, in batch-type or single-cavity rotors, and to a lesser extent in very large tubes (depending on concentration of material), the minor thermal gradients existing even with the rotor spinning in a fair vacuum, and to a lesser extent the minor fluctuations in speed, may cause the sedimentation behavior to approach what might be called "asymptotic sedimentation." This refers to a situation in which only the layer of fluid immediately adjacent to the wall or packed sediment remains stagnant. The main body of the fluid is constantly, though perhaps slowly, circulating and remixing so that the total amount of material being deposited in the stagnant zone per unit time is directly proportional to the concentration in the main body of the fluid. Thus, if a certain effective centrifugation time precipitates half of the starting material, doubling the time would produce 75 per cent separation and tripling the time would remove $87^1/_2$ per

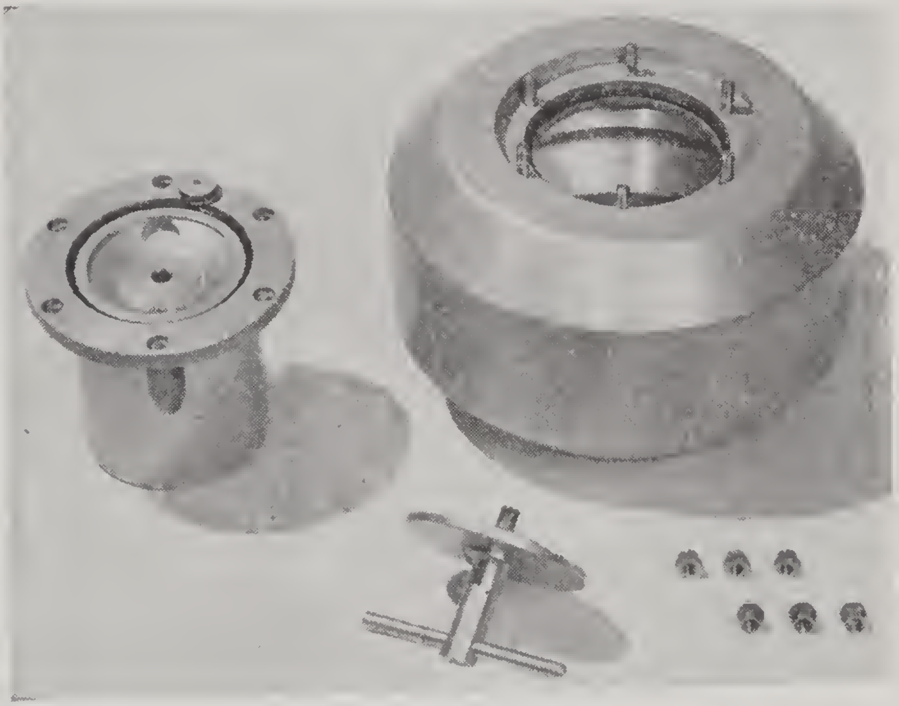

Fig. 9.—Hollowed-out batch-type rotor for the preparative ultracentrifuge (Fig. 7). Diameter is 9 in., capacity 1.7 l, speed 20,000 rpm. Capacity can be almost doubled in larger, slower rotors. (Courtesy of Specialized Instruments Corp.)

cent; but theoretically it could never be completely precipitated. However, computations for typical rotor designs and preliminary experiments have shown that $^2/_3$–$^3/_4$ of the material is removed in the time theoretically required for complete precipitation in the absence of convection, and hence with vacuum centrifuges equation (9) still furnishes a fair method of comparing rotor designs, and approximate precipitation times for known materials can still be predicted.

The Sharples continuous-flow supercentrifuge has been found useful in the concentration of some materials, particularly the larger viruses, and several articles (33, 38) describing its application have been published.

Note.—This section was reviewed by John Lawrence Oncley and Lena A. Lewis.

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SECTION II

Methods of Renal Study

ASSOCIATE EDITOR—*A. C. Corcoran*

INTRODUCTION

THE TIDE of publications on the function and structure of the kidney and on their interrelationships continues to swell. The interest which exists is shown by the fact that methods which only a few years ago were only of recondite and occasional use among the dwellers in the fabled ivory towers have become almost routines of scientific medicine. A scattering of specific procedures has been developed which fit only within the narrow bounds of an experimental protocol. We are not here as concerned with these as with methods which seem of wider application than is generally realized and a few so new that their promise is yet to be fulfilled.

Those selected are drawn from diverse disciplines and technologies. The methods described through page 213 are applicable in animal and clinical physiology. The study of renal tubular excretion in vitro (p. 228) is at the margin between physiology and biochemistry, while electron microscopy of the kidney (p. 234) is morphologic. The technique of endogenous creatinine clearance (p. 215) is an easily reproducible test of renal function which will be of interest to those engaged in the scientific practice of clinical medicine. The balance of the section is for those interested in experimental pathology in small animals, especially in rats.

The coverage is obviously incomplete and may be considered definitely spotty. Those who know the field will recognize that an orderly exposition even of a special topic might very well exhaust this and many volumes like it. If we have not erred in the process of selection, we have certainly strayed but, to our impression, in green pastures and beside the still waters. In brief, the aim is to present techniques which may find direct and wide application and others which open new directions in the study of the kidney.

—A. C. CORCORAN.

ESTIMATION OF RENAL FUNCTION FROM PLASMA DISAPPEARANCE RATES

MANY ATTEMPTS have been made to determine renal function from the rate of disappearance of intravenously injected test substances. Although mathematically attractive, most such estimates suffer from failures of rapid equilibration of rapidly decreasing peripheral venous plasma concentrations with body fluids on the one hand and simultaneously collected urine on the other. However, in appropriate circumstances, 3 methods can give useful approximations of excretion rates, especially of substances which are excreted relatively slowly.

Dominguez has developed the formulae which apply generally to disappearance of substances from the plasma by renal excretion, with and without simultaneous extraurinary loss. Robson's methods (p. 139) are aimed at clinical evaluation of specific renal functions. Differences in their symbols are:

SIGNIFICANCE	DOMINGUEZ	ROBSON
Excretion rate	y', y	uv
Plasma concentration	x', x	P
Clearance	A	C

Further, the symbols V, V_1, and V_2 in Dominguez' formulation represent constants: V in Robson's concept is an empiric function of time (t), explicitly $V(t) = a + bt$, written V_1 and V_2 to indicate the value of V at times t_1 and t_2.

I. Kinetics of Renal Excretion of Injected Substances

RAFAEL DOMINGUEZ, *St. Luke's Hospital, Cleveland*

The rate of excretion of several substances is a linear function of the plasma concentration of these substances. The general equation can be written as

$$y' = p + Ax' \tag{1}$$

where y' is the rate of excretion in mg/min, x' is the plasma concentration in mg/100 cc, and p and A are constants. This equation can always be reduced to

$$y = Ax \tag{2}$$

a form specially desirable when the values y' and x' include a blank or an endogenous value for the substance in question (1). If the substance is injected rapidly into the veins and is excreted completely by renal excretion, then, after diffusion between the blood and other body fluids has attained a steady state, the time curve of the plasma concentration is a simple exponential of the time

$$x = ae^{-\alpha t} \tag{3}$$

where $\alpha = \frac{A}{V}$, and V is the hypothetical volume of body fluid in which the substance is distributed in 1 fluid compartment at the same concentration as in plasma (2). The amount excreted in the time t_1 is

$$\int_0^{t_1} y\,dt = A \int^{t_1} x\,dt = A\,\frac{a}{\alpha}\,(1 - e^{-\alpha t_1}) \tag{4}$$

The assumption of complete excretion by the kidney makes the quantity excreted approximately equal to the amount injected G. If, in addition, the product αt_1 is so large that the term $e^{-\alpha t_1}$ can be neglected, equation (4) becomes

$$\int^{t_1} y\,dt = G = A\,\frac{a}{\alpha} \tag{5}$$

Therefore, when both utilization and diffusion are neglected, the 1st approximation (3) to the clearance of the substance by plasma data alone is given by

$$A_1 = \frac{G}{\left(\frac{a}{\alpha}\right)} \tag{6}$$

If diffusion into a 2d fluid compartment is taken into consideration (4), 2 exponentials may be sufficient to express the time change of the plasma concentration x

$$x = ae^{-\alpha t} + be^{-\beta t} \tag{7}$$

and also the time change of the concentration z in the 2d fluid compartment

$$z = c\,(e^{-\alpha t} - e^{-\beta t}) \tag{8}$$

with c given by

$$c = \frac{\beta a + \alpha b}{\beta - \alpha} \tag{9}$$

From equation (7) the 2d approximation A_2 to the clearance is obtained

$$A_2 = \frac{G}{\frac{a}{\alpha} + \frac{b}{\beta}} \tag{10}$$

If the substance is in part excreted by the kidney and in part metabolized (utilized), and the substance disappears exponentially from the blood stream, another equation is required

$$u = Bx \tag{11}$$

symbolizing the relationship of the rate of utilization u to the plasma concentration x, with B a constant (2).

In this case, the amount excreted E will always be less than the amount injected G, and the clearance A_3 is

$$A_3 = \frac{E_{t_1}}{\frac{a}{\alpha}(1 - e^{-\alpha t_1})} \tag{12}$$

leading to

$$A_3 = \frac{E_{t_1}}{\left(\frac{a}{\alpha}\right)} \tag{13}$$

if the period of collection t_1 is long enough (3).

In equations (12) and (13) utilization is taken care of, but not diffusion. If both utilization and diffusion are taken into consideration (3),

$$A_4 = \frac{E_{t_1}}{\frac{a}{\alpha}(1 - e^{-\alpha t_1}) + \frac{b}{\beta}(1 - e^{-\beta t_1})} \tag{14}$$

an equation which simplifies to

$$A_4 = \frac{E_{t_1}}{\frac{a}{\alpha} + \frac{b}{\beta}} \tag{15}$$

if t_1 is long enough.

The error made in A_1 by neglecting diffusion is determined by the ratio

$$\frac{A_1}{A_2} = 1 + \frac{\alpha b}{\beta a} \tag{16}$$

The error made in A_1 and in A_2 by assuming no utilization is determined by the ratios

$$\frac{A_1}{A_3} = \frac{A_2}{A_4} = \frac{G}{E} \tag{17}$$

Finally, the error introduced in A_1 by neglecting both diffusion and utilization is

$$\frac{A_1}{A_4} = \frac{G}{E}\left(1 + \frac{\alpha b}{\beta a}\right) \tag{18}$$

The volumes of distribution of the substance associated with A_1 and A_2 (5) can be computed from the equations

$$V_1 = \frac{G}{a} = \frac{A}{\alpha} \tag{19}$$

$$V_2 = \frac{G(\beta^2 a + \alpha^2 b)}{(\beta a + \alpha b)^2} \tag{20}$$

The error made in V_1, by neglecting diffusion before the steady state, can be computed from the ratio (3)

$$\frac{V_1}{V_2} = 1 + 2\frac{\alpha b}{\beta a} \text{ approx.} \tag{21}$$

Note.—Many of the equations given here have been tabulated without development in reference (3). The derivation of the equations for substances which penetrate 2 fluid compartments are to be found in reference (4). A review of this work with extension to intestinal absorption and a survey of volumes of distribution is presented in reference (5).

Comment by James S. Robson

V (of Dominguez) is taken as the "volume of body fluid in which the substance is distributed at the same concentration as in the plasma" and is assumed to be constant in the derivation of formula (6). The assumption is justified when equilibration of injected material between plasma and extracellular fluid is attained at the instant of injection and thereafter maintained. If, however, equilibration occurs at some time after injection, and a steady state is subsequently maintained, the assumption is justified only from that time, and formula (14), incorporating corrections for loss before equilibration and for utilization or destruction in the body becomes valid, while formula (6) ceases to apply.

However, following single intravenous injections of diodone or PAH in amounts suitable for estimations of renal blood flow, and of inulin in amounts which provide plasma concentrations adequate for estimation of glomerular filtration (Robson, ref. (9) and (10) in this volume, p. 145), the paired constants a and α and b and β cannot be separated at any time at which accurate clinical analyses are feasible. The large molecular size of inulin and the protein-binding of diodone and PAH presumably prevent equilibration within the desired limits of time. The smaller molecular size of mannitol appears to permit attainment of equilibration after a single intravenous injection, so that Dominguez' equations can be directly applied, which they cannot be in the former cases. It is possible that by giving larger single injections of inulin than used hitherto, equilibration might be attained before the substance disappeared from the plasma, although this remains to be tested.

Because of these facts, the formulae of Robson *et al.* were devised. In these, the volume of distribution is used empirically as a function which varies with time, and the experimental conditions in the cases of inulin, diodone and PAH are such that the function increases at an approximately constant rate between defined limits of time following injection.

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II. Single Injection Technique in Evaluation of Renal Function

JAMES S. ROBSON, *University of Edinburgh*

Single injection methods of evaluating discrete renal functions in human subjects have been suggested by numerous workers, some of whom have attempted to simplify the technique of Smith, Goldring and Chasis (13). Simple slope analysis which takes into account solely the rate of disappearance from plasma of injected material (1, 2, 5) has been shown to be invalid for most substances. Such analysis assumes, among other things, instantaneous and maintained equilibration of the injected substance in its volume of distribution. In the case of mannitol, for example, this equilibration is attained only after a significant renal and extrarenal loss (3). In the case of inulin, diodone, i.e., iodopyracet, Diodrast (9, 10) and PAH (11), equilibration does not occur during the several hours immediately following a single intravenous injection.

Factors which make allowance for failure of equilibration may be so incorporated into the slope analysis as to permit calculation of renal functions. Estimations of rates of renal excretion by the collection of urine over short periods of time during intravenous infusion can be avoided.

MEASUREMENT OF RENAL CLEARANCES (EXCRETION CONSTANTS) OF INULIN, DIODONE AND PAH

Following a single intravenous injection of inulin, diodone and PAH in amounts suitable for the determination of their respective clearances, their plasma concentrations fall with time from the instant of injection. Meanwhile, their volumes of distribution as defined by Robson *et al.* (9–11) continue to rise. This is because plasma concentration does not represent the average extravascular concentration; indeed, the latter changes at a rate which is slower than the rate of change of plasma concentration (9–11). In these circumstances

$$\frac{dX}{dt} = -uv = \frac{dPV}{dt} \tag{1}$$

where X = the amount of substance in the body; P = plasma water concentration of the substance; V = volume of distribution and is $\frac{X}{P}$, and uv = rate of loss of substance in the urine.

It may be shown that V increases approximately linearly with time for a considerable period after the injection of inulin, diodone or PAH. Equation (1) may be written

$$-uv = (a + bt)\cdot\frac{dP}{dt} + Pb$$

where $V = a + bt$, in which a and b are constants. Therefore

$$-C = b + \frac{(a + bt)}{P}\cdot\frac{dP}{dt} \tag{2}$$

where $C = \frac{uv}{P}$ and represents plasma water clearance and is assumed to be constant. Solving formula (2) and integrating between 2 times, t_1 and t_2,

$$C = \frac{b(\log_{10} P_1 - \log_{10} P_2)}{\log_{10} V_2 - \log_{10} V_1} - b \tag{3}$$

RENAL CLEARANCE OF INULIN (8)

a) Preparation and injection of solution.—Inulin (Kerfoot's pure bacteriological) is dissolved in sterile saline with heat to make an approximately 10 per cent (w/v) solution, and the solution is passed through a sterile Seitz filter. It is then suitable for injection. The inulin content of each batch of solution is determined chemically.

A sample of venous blood (for blank analysis) is withdrawn from the subject and an accurately measured volume of the inulin solution, containing 100–120 mg of inulin/kg of body weight, is injected intravenously (a syringe of 100 ml capacity is convenient), the injection taking 2–5 min. The time is noted when the injection begins and ends. With this technique, no evidence of toxicity has been observed.

b) *Blood and urine collection.*—At 2 noted times (t_1 and t_2) about 30 and 100 min after the midpoint of the injection, urine is collected, the bladder being emptied as completely as possible. Since it is necessary to know only the total amount of inulin excreted, there is no need for the subject to empty the bladder before the injection; on the contrary, a full bladder at that time increases the accuracy of the first urine collection 30 min later. Two to 3 min before these urine collections, venous blood is withdrawn (10 ml).

c) *Calculation.*—The weight of inulin injected minus the weight of inulin in the urine passed at the first collection (t_1) gives the weight of inulin remaining in the body at that time. This, divided by the plasma water-inulin concentration P_1, taken 2–3 min before this collection, gives the first volume of distribution V_1. Similarly, the weight of inulin injected minus the total weight excreted in both urine samples gives the weight remaining in the body at the time of the 2d urine collection (t_2), and this, divided by the second plasma water concentration of inulin P_2, gives the second volume of distribution V_2.

C is readily converted to the conventional plasma clearance of inulin (C_{In}) in ml of plasma/min by multiplying by the factor, $100/(100 - \text{g protein}/100 \text{ ml plasma})$.

RENAL CLEARANCE OF DIODONE AND PAH

The method is essentially similar to that for inulin clearance and so also is the calculation. $C \times 100$ yields estimated plasma clearance.

a) *Preparation and injection of solutions.*—It is convenient to use, without dilution, the 35 per cent (w/v) sterile solution of diodone obtainable commercially in sterile ampules. The dose required is one which will give plasma-diodone levels of between 5 and 1 mg of diodone-iodine/100 ml of plasma for the period of the urine collection. This requires 7–8 ml of solution for a person of 70 kg body weight and normal renal function; when severe depression of renal function is expected, the dose should be reduced to 4 ml for 70 kg body weight. The injection should take 1–2 min, the exact times being recorded. Toxic reactions to these small amounts of diodone are uncommon; they include urticaria, tenesmus, flushing and vomiting.

Solutions of PAH may be purchased or prepared by dissolving

PAH acid in normal NaOH, carbonate-free, with subsequent adjustment of the pH to 7.2. Three grams may easily be dissolved in 15 ml. The solution is then passed through a Seitz filter and autoclaved. The dose required is one which will give plasma diodone-iodine concentrations between 5 and 10 mg/100 ml for the duration of the test. This requires 9–12 ml of the above solution for normal people of 70 kg. The amount should be reduced if depression in renal function is suspected. The injection should take 2–5 min. Toxic effects, including vomiting occasionally occur.

b) Blood and urine collection.—The procedure is similar to that after inulin injection, but because of the rapid elimination of diodone and PAH the first blood and urine collections should be made 20 min, and the second 60 min, after the midpoint of the injection.

c) Calculation.—For the respective collection times t_1 and t_2 (measured in minutes from the midpoint of the injection), the volumes of distribution V_1 and V_2 are calculated exactly as for inulin. P_1 and P_2 are the concentrations of diodone-iodine or PAH in mg/100 ml of plasma water at times t_1 and t_2. The constants of equation (3) and the clearances in terms of water or plasma are also calculated as for inulin.

MEASUREMENT OF MAXIMAL TUBULAR EXCRETION OF DIODONE

A method of analysis similar to that used for inulin and diodone clearance may be applied to estimate the maximal tubular excretory power for diodone (10). Following a single intravenous injection of diodone large enough to saturate tubular excretory capacity for the period of urine collection, the plasma concentration falls and the volume of distribution rises from the instant of injection. Accepting maximal excretory capacity as a constant, the basic formula of Smith *et al.* (13), rearranged, is

$$uv = \mathrm{Tm_D} + \mathrm{FWC_{In}P}$$

where $\mathrm{FWC_{In}P}$ = the filtered diodone (14). Employing symbols similar to those used in the preceding section

$$-\frac{dX}{dt} = (\mathrm{Tm_D} + \mathrm{FWC_{In}P})$$

Now, $X = P \cdot V$. Therefore

$$\frac{dX}{dt} = P\frac{dV}{dt} + V\frac{dP}{dt}$$

Therefore

$$\mathrm{Tm_D} + \left(\mathrm{FWC_{In}} + \frac{dV}{dt}\right)P = -V\frac{dP}{dt} \qquad (4)$$

Under the conditions of the test it may be shown that V varies linearly with time. Therefore

$$\mathrm{Tm_D} + (\mathrm{FWC_{In}} + b)P = -(a + bt)\frac{dP}{dt}$$

Solving this for P and integrating between times t_1 and t_2,

$$\mathrm{Tm_D} = (C' + b)\,\frac{P_2V_2^{\frac{C'+b}{b}} - P_1V_1^{\frac{C'+b}{b}}}{V_1^{\frac{C'+b}{b}} - V_2^{\frac{C'+b}{b}}} \qquad (5)$$

where $C' = \mathrm{FW^2C_{In}}$.

PROCEDURE (8)

a) Injection of diodone.—The required amount of diodone ranges from 350 to 200 mg/kg of body weight according to the (expected) renal efficiency of the subject: the greater the efficiency the greater the dose. The solution used, 50 per cent diodone (w/v), is obtainable, sterile, in ampules, and an average-sized normal subject is given 30–50 ml at a rate not exceeding 5 ml/min.

When given in these quantities, diodone occasionally produces side-reactions which include nausea, vomiting, tenesmus, colic, urticaria, faintness, fall in blood pressure, itching and pain over the injection site. Incidence and severity of such reactions are, however, minimized by the prescribed slow rate of injection.

b) Collection and analysis of samples and calculation.—Measuring time, as usual, from the midpoint of the injection, blood and urine are collected after approximately 30 and 100 min, V_1 and V_2 are calculated, using P_1 and P_2 as described, and thence, as before, the constant b.

P_1 and P_2 are the plasma water concentrations of diodone-iodine in mg/100 ml. The value for F is obtained from the nomogram of Smith and Smith (14), and W^2, which is the square of the water fraction of plasma, is estimated from a knowledge of the plasma protein concentration. Equation (5) may then be solved for Tm with a knowledge of the value of inulin clearance. This is preferably determined simultaneously with the data needed for solution of equation (5), the injection of inulin can be given immediately before the injection of diodone. Estimations of both compounds may be made on the same blood or urine samples. Alternatively, the 2 determinations may be made on 2 separate occasions.

EVALUATION OF ERRORS INVOLVED IN SINGLE INJECTION METHODS

It is fair to say that methods of determining discrete renal functions employing continuous intravenous infusion and collection of urine by catheter over short periods (10–15 min) are freer from theoretical objections and the possibility of systematic errors than single injection techniques. Nevertheless, any method which extends the possible period of urine collection to 60–80 min succeeds in eliminating the greatest single source of technical error in such determinations, namely, the direct estimation of excretory rates by sampling of voided urine over short periods.

In practice, the differences between results of the methods described and those involving continuous infusion with short period urine collection are insignificant as far as inulin clearance and maximal tubular excretory capacity are concerned (6). This is because arteriovenous differences in concentration of inulin and diodone with single intravenous injections of appropriate magnitude are insignificant for inulin (4) and of the order of 5–10 per cent for diodone (11). Agreement between estimated diodone and PAH clearances as determined by the 2 methods is not as good; the infusion method gives results which average 20 per cent higher than those obtained by single injection. The arteriovenous difference of diodone concentrations in these circumstances varies from 7 to 25 per cent and is apparently roughly proportional to the level of renal blood flow (6). Inequality between peripheral venous and mixed arterial concentrations is largely responsible for the disparities in clearance values obtained by the 2 methods. This disadvantage of the single injection method can be overcome either by sampling arterial blood or by setting up an arbitrary range based on venous sampling at various levels of renal blood flow. Development of micromethods applicable to capillary blood might effectively replace the former. Still, venous sampling permits analysis of data on values thus derived which correspond with similar data obtained by infusion techniques at least in all types of abnormal renal conditions which have been studied (7, 11).

There have been other recent objections to the single injection technique (12). Variations in the volume of extravascular fluid over the period in which the formulae are applied do not affect the validity of the method, in the sense that they yield values of clearance which are the average for the time over which they are calculated. Abnormalities in the value for the volume of distribution (which has no real physiological significance in terms of fluid compartments) which might be produced by circulatory failure or oedema, do not disturb the validity of formula (1) upon which the subsequent derivations are based. The method is independent of the attainment of any steady state between plasma and extravascular compartments and is, as a consequence, not affected by the rate of mixing between these compartments.

There does remain, however, the possibility that error might arise from the difficulty in relating any particular amount of excreted substance to a plasma concentration which is changing with time (12). This does not appear to be significant in the case of inulin clearance and maximal tubular excretory power (6). Its effect is likely to be minimized, moreover, when long periods of urine collection are related to a large segment of the plasma concentration-time curve, for the reason that increments or deficits in the excreted substance which might result from such a cause will be smaller in relation to the total collected than with short collection periods. Nevertheless, it seems likely that a real and significant error is introduced into all single injection methods in which rapid change in plasma level occurs, i.e., in determinations of diodone and PAH clearance, as a result of this failure to sample representative urine. The magnitude of this error is unknown. It is, however, not likely to be large since results of single injection clearance methods closely approximate those obtained by infusion methods when arterial blood is sampled and when the customary 2–3 min allowance is made for delay or minimal appearance time.

NOTE.—This section was reviewed by Rafael Dominguez.

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RENAL BLOOD FLOW

I. Extraction and Clearance Method

JAMES F. NICKEL *and* STANLEY E. BRADLEY, *Columbia University*

The use of sodium p-aminohippurate or Diodrast clearance for the measurement of renal plasma flow is based on the assumption that these substances are completely cleared from the plasma perfusing the kidneys. When renal extraction of PAH or Diodrast is impaired by tubular dysfunction or by competitive interference by other substances excreted in a similar fashion, the clearance procedure alone is insufficient for this purpose and the actual amount of PAH or Diodrast removed from each volume of plasma flowing through the kidneys must be measured directly. Development of the venous catheterization technique by Cournand and his associates (2) has made this possible in man and animals under physiologic conditions.

PROCEDURE

Under local anesthesia,* through a small incision a radiopaque ureteral catheter is introduced into a medial antecubital vein and passed under fluoroscopic control by way of the superior vena cava, right atrium and inferior vena cava into the right renal vein. (The right renal vein must be used because the ovarian or spermatic veins empty into the left renal vein and peripheral venous blood is mixed with renal venous blood.) Isotonic saline or glucose solution is allowed to flow slowly through the catheter while it is kept in place. To avoid dilution by the infusion fluid 2 or 3 ml of blood is withdrawn and discarded before a sample is taken for analysis. As a rule, blood samples to serve as blanks are taken simultaneously from a peripheral vein and the renal vein before determination of clearances. Clearances are measured as described by Selkurt (3) and renal venous blood is sampled simultaneously with peripheral venous blood.

The total renal plasma flow may then be calculated by dividing the amount of the material excreted in the urine each minute, UV, by the difference between the concentrations in each milliliter of peripheral and renal venous plasma or by dividing the clearance value C, by the renal extraction ratio E (the concentration difference between peripheral and renal venous blood divided by the peripheral concentration). It can be seen that any substance which is extracted from the blood by the kidney and excreted as such may be used for this purpose. Inulin, mannitol and urea as well as PAH and Diodrast have been employed at various times. Diodrast is seldom, if ever, used because it enters the erythrocytes and diffuses out into the plasma once it has been removed from the plasma by the renal tubule cells. Rapid separation of plasma and red blood cells may circumvent this difficulty, but it is preferable and more convenient to use PAH in man because it is not taken up by the erythrocytes and is easily determined chemically. There is no evidence that PAH is appreciably acetylated or metabolized by the human kidney (1).

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* Procaine should not be used if PAH extraction is measured because it may interfere with the chemical determination of PAH.

Comment by J. K. Clark

The technical details of renal venous catheterization are much the same as those of other catheterization methods (4). The patient should be supine. Since catheters sterilized by boiling soften, they may be stiffened in iced saline before use. Venous spasm which may interfere with effective catheter manipulation can often be relieved by such means as stopping movement of the catheter, warming the arm, infiltrating a local anesthetic (choosing one which will not interfere with chemical determinations) along the vein, withdrawing the catheter and reinserting it after an interval or inserting a smaller catheter.

On insertion the catheter may tend to pass into the neck, the right ventricle or a hepatic vein, so that passage into the right renal vein is a matter of trial and error based on experience and confidence. Entry into the vein is usually obvious fluoroscopically. Respiration and softening of the catheter may result in displacement, so that the position should be checked as the experiment ends. Difficulty in withdrawing blood may result from entry into one of the small branches of the renal vein or pressure of the catheter against the vein wall. Careful retraction under observation will correct this. Deep inspiration sometimes facilitates sampling but may lead to displacement and alter the results of gas analysis.

Catheters through which many samples are to be taken are kept patent by adding a small amount of heparin to the saline drip. Since renal venous blank values for inulin, mannitol and PAH are identical with peripheral arterial or venous blood, time can be saved by taking peripheral blood for the blank and starting the infusion of test solution before catheterization begins. The desired stable plasma concentrations are thereby attained while the catheter is being passed.

The vein need not be tied off after withdrawal. A pressure dressing will control bleeding and an adhesive "butterfly" is used for skin approximation. Patients should be warned to expect some stiffness and discomfort from thrombosis. To my knowledge, there has never been a serious complication of renal venous catheterization and there seems to be no absolute clinical contraindication to this procedure. However, we have avoided its use in subjects with myocardial irritability or a tendency to thrombosis.

Contrary to earlier impressions, recent evidence indicates that PAH may penetrate red cells in man. In a communication to Dr. H. G. Barker, Dr. R. A. Phillips states that although the process is slow, it is nevertheless definite, and in unusual circumstances, such as elevation of plasma PAH concentration for measurement of Tm followed by a fall in concentration, diffusion from red cells may invalidate the use of plasma concentrations in the Fick equation. Conn and Markley have matched Fick PAH determinations against direct (bubble flow meter) measurements of renal blood flow and have demonstrated the validity of the equation as applied to whole blood. Unfortunately, determination of PAH in whole blood is less satisfactory than in plasma. Under usual conditions, especially if plasma is separated with reasonable promptness, red cell diffusion of PAH should introduce little error into estimates of blood flow based on plasma determinations, since a false doubling of the renal venous estimate will decrease apparent blood flow by less than 10 per cent at normal levels of extraction ratio. This factor becomes serious as extraction falls.

Comment by A. C. Corcoran

Wolf (5) pointed out that renal extraction of water should be allowed for in calculations of renal blood flow based on renal arteriovenous difference. This allowance increases at high levels of urine flow, as during osmotic diuresis, and when the extraction ratio measured is small. However, in general, such errors are small as compared with technical inaccuracies. Thus, in the case of inulin, 5 per cent errors in opposite senses in arterial and venous plasma concentration estimates will alter the estimate of extraction from 0.2 (true) to 0.28 or 0.105 (apparent); at an inulin clearance of 130 cc/min, the true renal plasma flow of 650 will be recorded respectively as 465 or 1,240 cc/min. The importance of scrupulous technique and of basing estimates of blood flow on substances of high extraction is therefore apparent.

The technique of determination of renal blood flow by measurement of clearance and extraction is described not only because of its intrinsic importance in the study of renal blood flow in the diseases of human beings but because the process of catheterization of the renal vein will probably come into still wider use in the study of renal metabolism.

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II. Methods for Direct Measurement

EWALD E. SELKURT, *Western Reserve University*

One of the desirable attributes of a directly recording flow meter would be that it offer minimal resistance to blood flow so that nearly normal pressure relationships would be obtained whether used in series with the artery (arterial inflow method) or vein (venous outflow method). Although the optically recording bubble flow meter is unsurpassed for simplicity and precision (4), it suffers from the disadvantage of not being continuously recording and, in the range of renal blood flows, causes considerable pressure drop because of its resistance, hence making it unsuitable for venous outflow measurement. To obtain continuous recording of renal blood flow with minimal pressure loss, use has been made of a large size optically recording rotameter originally designed for direct measurement of cardiac output in dogs (2, 6).

ROTAMETER

This rotameter has a diameter of 30 mm across its chamber and contains a float of 22 mm diameter. Different ranges of flow are obtained by using floats of different weights: Lucite, 0–500 cc/min; Lucite with brass slug, 0–900 cc/min; all brass float, 0–1,500 cc/min. The 1st range finds best application for renal work. Pressure drop across the rotameter and connections has been found to be about 4 mm Hg at 100 cc/min, and about 9 mm Hg at 200 cc/min. The low resistance of this meter makes it permissible to use it as a venous outflow recorder as well as for arterial input metering. Cannulation of the vein can be direct, but it has been used in connection with indirect cannulation of the left vein via the right jugular vein and inferior vena cava. A suggested modification of the original method (3) is to use plastic tubing of 4 mm ID and 5 mm OD guided into the vein by a loose-fitting stilet (1). Blood passing out through the rotameter is passed directly into the opposite jugular vein. By projection of the return cannula into the superior vena cava, advantage can be taken of the negative intrathoracic pressure to compensate to a large degree for pressure drop across the circuit.

PROCEDURE

Connections for using the rotameter for arterial inflow measurement as used in this laboratory are shown in Figure 1. The left renal artery is prepared by careful separation of nerve fibers. Its axis is ligated at the junction with the abdominal aorta and the cannula is directed toward the kidney, which is then perfused through an external circuit of Tygon tubing from the carotid artery. The rotameter is in series with this circuit. The heparinoid Paritol* has been used successfully as an anticoagulant in initial dosage of 25–50 mg/kg intravenously, with booster doses of 50 mg total every half-hour. A pump† is used to obtain a wide range of perfusion pressures and, in connection with 2 damping chambers, D_1 and D_2, also provides a wide range of pulse pressures. The advantage of this circuit is that the rotameter can be calibrated at any time by supplying the kidney by an alternative circuit from the femoral artery by opening clamp *9* and closing clamp *8*. In the meantime blood is passed through the rotameter and shunted back through the femoral vein at varying rates dependent on the setting of clamp *7*. For calibration after flow has stabilized, about 10 cc is shunted into a cylinder from a 3-way stopcock, *S*, with elec-

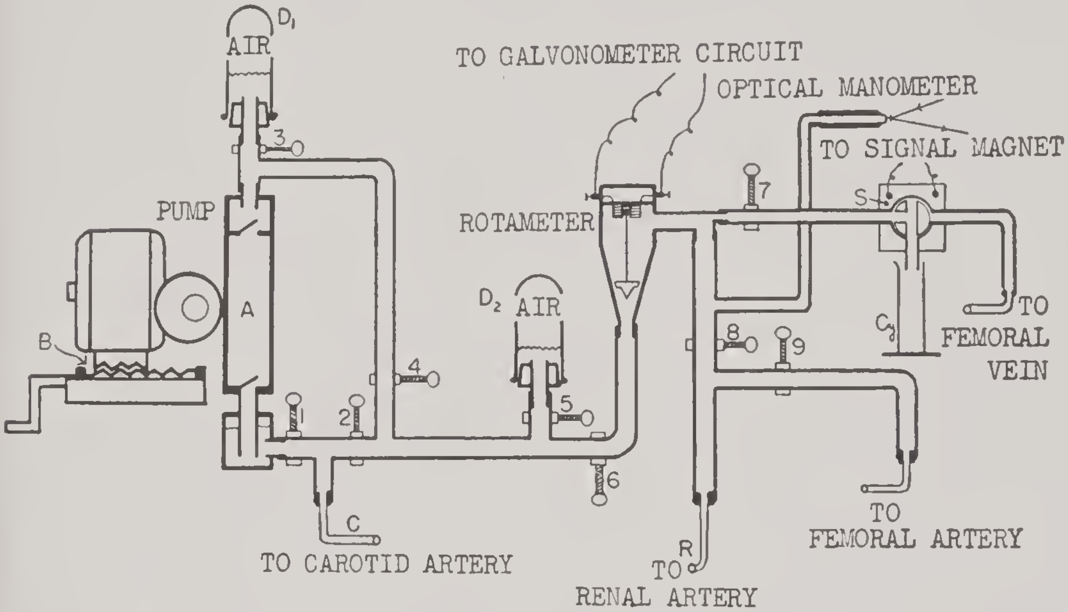

FIG. 1.—Perfusion circuit for measurement of direct renal blood flow using rotameter principle with provisions for varying mean perfusion pressure and pulse pressure. Rotameter calibration is achieved by shunt circuit to the femoral vein and an electrically recording 3-way stopcock, *S*. During calibration the kidney is supplied from the femoral artery (*lower right*) by opening clamp *9* and closing clamp *8*. Calibration flows are varied with clamp *7*. Pump chamber, *A*, is made of Elastomold tubing of 3.5 cm OD and 12 cm length and supplied with rubber flap valves at top and bottom. It is actuated by a rotating ball-bearing cam driven by a Bodine motor turning at 120 rpm. Motor is mounted on a worm gear, *B*, so that stroke volume can be altered. D_1 and D_2 are air damping chambers. Clamps *1* and *4* are used to close off the pump circuit; clamps *2* and *6* aid in adjustment of mean and pulse pressures; clamps *3* and *5* regulate the air chambers.

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* Supplied by Dr. Joseph Seifter, Wyeth Institute of Applied Biochemistry, Philadelphia.

† The pump was constructed by Mr. Clifford Wilson, 325 W. 42d St., Indianapolis, Ind.

trical contacts for precise recording of the time of outflow by means of a signal magnet registering on the optical record.

Critique.—Difficulties encountered in use of this rotameter have been the result of the nonlinear character of the calibration curve, necessitating numerous determinations for best accuracy, and the tendency of the curve to drift. The former is due to inherent characteristics of the electrical circuit, and the drift is due to temperature change. Changes in blood viscosity, possible fibrin formation and changes in line voltage may be other sources of difficulty. As pointed out by Shipley, the instrument is a mean flow recorder and is capable of quantitating pulsatile flows with reasonable accuracy only when the float does not hit the top or bottom at the extremes of its excursion, and only when entering fluid is not directed as a jet into the bottom of the instrument. The latter occurs when adapters or tubing smaller than the inflow spout is used to convey blood to the rotameter.

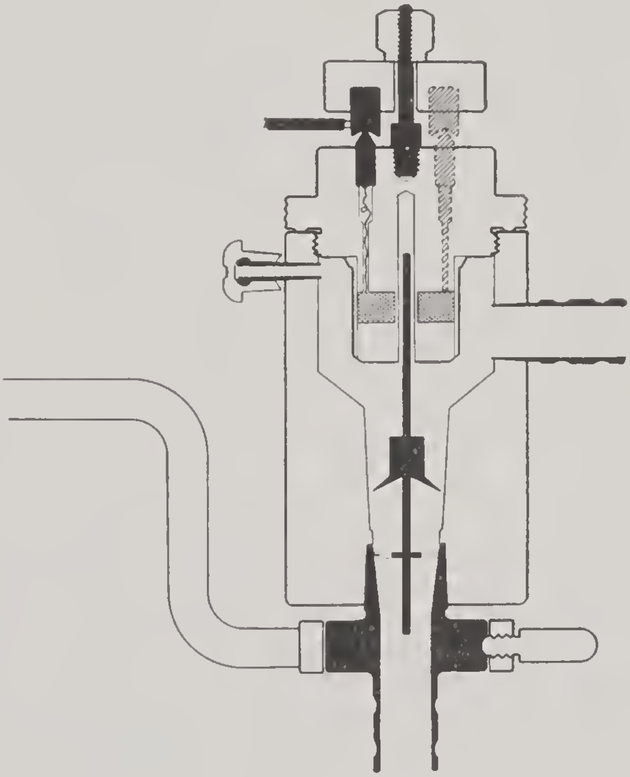

FIG. 2.—4–S model recording rotameter, 400 ml capacity. (Courtesy of Shipley and Wilson (5).)

The latest type model, 4–S series (Fig. 2), designed by Shipley and Wilson (5) attempts to minimize some of these disadvantages of the older models. The improved features of the new series are:

1. A standard detecting top portion which may be used with

interchangeable bottom metering portions having different flow ranges.

2. Inclusion in the detecting top portion of a noninductively wound coil (made of the same wire as the detecting coil) which serves as the opposing resistance arm of the bridge. This change essentially eliminates any drift due to changes in blood temperature causing change in coil resistance.

3. Use of manganin or Advance wire wound resistors inside the control box for the other 2 arms of the bridge. Additional thermal drift is eliminated thereby.

4. Widening of the Lucite chamber in which the float rides so that for all metering bottoms the pressure drop required to just lift the float is between 1.5 to 2.0 mm Hg. Pressure drop across the entire instrument is estimated to be only 2.0–2.5 mm Hg at maximal flow.

5. Redesign of the float disk so that changes in viscosity of the blood have a minimal effect on the height of the float.

6. The calibration curve represents a better approximation to linearity in that it is the central or least curvilinear portion of a sigmoid curve.

Comment by R. E. Shipley

In common with other types of direct flow measuring devices the recording rotameter causes a relatively small but definite hindrance to the flow of blood. By increase of the bore of the rotameter and diameter of the float the pressure drop across the instrument could be reduced to an almost negligible value; however, the increased volume of blood required to fill the enlarged rotameter chamber might constitute a disadvantage in some experiments.

As a supplement to Dr. Selkurt's suggestions, it should be stressed that the accessory conducting tubing, connections, cannulae, etc., be made as short as possible and of maximal bore since the resistance to flow through the entire circuit determines the pressure drop and therefore the extent to which the flow of blood will be hindered.

Comment by A. C. Corcoran

The procedure described here is in a sense supplementary to Dr. Selkurt's contributions to this topic in Volume 1 of this series (pp. 191 ff.). However, the method has been developed largely since that time; as the characteristics of rotameters become more widely understood, the ingenious instruments are being put to use far afield from the kidney.

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WATER AND ELECTROLYTE METABOLISM

Introduction

A. C. CORCORAN

The techniques described here are so diverse that they fall under one heading only by a rather generous interpretation of the function of classification. Deane's description of infusion technique should perhaps have followed Dominguez' and Robson's contributions on the estimation of function from plasma disappearance rates had it not run so naturally into and along with his paper on body water compartments (space techniques). The latter supplements parts of the section on Fluid and Electrolyte Distribution edited by Flexner in Volume 4 of this series; it also carries on very naturally into the discussions by Wesson and Brodsky of the relationships between kidney function and the fluid, electrolyte and nonelectrolyte masses of the body. Experiments can readily be visualized which would incorporate some of Deane's methods with some of Wesson's studies in the dog. Brodsky's contribution is in part complementary to Wesson's discussion of osmotic diuresis and in part a new approach to the study of renal function in the diseases of human beings.

The transition from osmotic diuresis to the assay of diuretic and antidiuretic substances is perhaps easier etymologically than physiologically. The contributions by Little, Turner and Grollman, and Gaunt form substantially as complete a survey of this still vexed field as could be gathered.

Renal excretion of water and electrolytes can be assessed either in terms of over-all body economy as, for example, in the studies elaborated by A. V. Wolf in his excellent text, *The Urinary Function of the Kidney* (2), or as discrete and isolatable functions of nephrons and parts of nephrons. Both approaches have excellencies and advantages, the latter illustrated by Homer Smith in *The Kidney* (1).

Among discrete functions, the excretions of water and salt are of the first interest, and perhaps the greatest complexity, and their exploration is only beginning. For these reasons, Wesson and Brodsky were invited to develop their procedures and concepts in some detail. In so doing, they have brought to light aspects of conceptual disagreement which future studies will resolve. These arise in part from inherent differences in their aims. Wesson's are more academically and Brodsky's more clinically oriented. Wesson's hope, and ours, is not as much to encourage exact repetition of his carefully controlled experiments as to enable the thoughtful reader to proceed with modifications and elaborations. Brodsky and his colleagues have specified a measurable aspect of kidney function which can be studied in dog and man, while Wesson points out that the rigors of urea diuresis can hardly find application in human beings. Both agree that the application of the methods they describe to studies of renal responses to drugs and to loads, in health and disease, have yet to be developed. The methods are presented not as definitive procedures with settled applications but as illustrations of a new and important direction in renal physiology.

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I. Infusion Technique for Measurement of Renal Function

NORMAN DEANE, *New York University*

Earle and Berliner (3) have described an infusion technique for measurement of renal function without bladder catheterization or collection of urine. Comparisons of the infusion and urine collection methods have been presented by Berger, Farber and Earle (1), who carefully defined the principle on which the procedure is based: "For any exogenous substance which is neither metabolized, nor excreted otherwise than in urine, the rate of excretion must be equal to the rate of infusion under conditions where its plasma level and volume of distribution are constant. This steady state is designated infusion equilibrium."

At the time of infusion equilibrium, the rate at which the substance is being excreted, UV (mg/min), is equal to the rate at which it is being injected, IV (mg/min), and the clearance calculation can be stated

$$C_x = \frac{I_x V}{P_x}$$

where C_x = clearance of x (cc/min); I_x = concentration of x in infusion (mg/cc); V = rate of infusion (cc/min), and P = plasma concentration of x (mg/cc). Inulin and p-aminohippuric acid clearances may be determined by this technique.*

PROCEDURE FOR INULIN AND PAH

1. A blood sample is obtained for determining plasma blank, and priming injections of inulin and PAH are administered. The priming injections are calculated as follows:

$$\begin{matrix}\text{Priming in-}\\ \text{jection (mg)}\end{matrix} = \begin{bmatrix}\text{wgt of sub-}\\ \text{ject (kg)}\end{bmatrix}\begin{bmatrix}\text{vol of distribu-}\\ \text{tion (\% wgt)}\end{bmatrix}\begin{bmatrix}\text{intended plasma}\\ \text{concn. (mg/cc)}\end{bmatrix}\begin{bmatrix}1{,}000\end{bmatrix}$$

For inulin, a volume of distribution of 18 per cent and a plasma concentration of 20 mg per cent may be used. For PAH, a volume of distribution of 28 per cent is employed and an intended plasma concentration of 2 mg per cent is used for measurements of effective renal plasma flow.

2. A sustaining infusion of inulin and PAH is begun (preferably through intravenous plastic tubing (4)). For measurements of glomerular filtration rate and effective renal plasma flow, the calculation is

$$\begin{matrix}\text{Concn. in sustaining}\\ \text{infusion (mg/cc)}\end{matrix} = \frac{\begin{bmatrix}\text{plasma concn.}\\ \text{(mg/cc)}\end{bmatrix}\begin{bmatrix}\text{estimated clearance}\\ \text{(cc/min)}\end{bmatrix}}{[\text{rate of infusion (cc/min)}]}$$

It is extremely important that the infusion rate be kept very constant and be known accurately.

3. After the infusion has continued for 4 hr, a minimum of 3 blood samples is collected at 30 min intervals, and the infusion is concluded.

Analytical technique.—The determination of inulin by the resorcinol method is described on page 163. Determination of inulin by the diphenylamine reagent and the analytical procedure for PAH have been presented by Selkurt (5).

In the infusion method for measurement of renal function, it is necessary to determine "total" plasma PAH after hydrolysis, since PAH is conjugated in man. This is done by adding 1 cc of 1.1N HCl to 10 cc of plasma filtrate and heating for 1 hr in a boiling water bath. One cc of N HCl is added to 5 cc of the hydrolyzed filtrate and the color reaction then performed in the usual manner.

Calculation.—The clearance is determined, at the time of infusion equilibrium, as the rate of infusion (IV) divided by the plasma concentration.

Critique.—If infusion equilibrium as defined by Berger, Farber and Earle (1) is reached, the infusion method is valid for measuring renal function. Inaccurate data are obtained if meticulous attention is not directed to the following details and limitations of this technique.

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* Tm_{PAH} may be measured by the infusion technique. However, the large amount of PAH required makes this procedure impractical; therefore the urine collection method is recommended for measurements of Tm.

The infused substance must have reached a maximal volume of distribution before calculation of clearance is made, and the plasma concentration must be constant. Thus, it is not practical to attempt application of this method in instances of increased extracellular fluid volume.

The composition of the infusion and rate of infusion must be known exactly. An independent check on the rate of the pump, similar to that presented for the calibrated infusion method for measurement of extracellular fluid (2), should be maintained throughout the period of infusion. An inconstant pump rate invalidates the method.

The use of substances which are not biologically inert (i.e., mannitol) invalidates the method.

The method should not be used for quantitative studies relating to acute changes of renal function. Calculations made by the infusion method indicate the direction but not the actual extent of sudden alterations in clearance (1). The discrepancy between the infusion and the urine collection methods will exist until the change in the concentration which will be effected by the change in clearance has been communicated throughout the volume of distribution of the substance.

Comment by David P. Earle, Jr.

The importance of strict adherence to the technical details of this method cannot be overemphasized. If the concentration of the substance whose clearance is being measured varies by more than 5 per cent in the 3 plasma samples, infusion equilibrium has not been achieved and the results should be discarded. Unfortunately, when renal function is less than 60 per cent of normal, the infusion technique may yield erroneously high values even though the plasma levels may indicate an apparent infusion equilibrium. This may occur when the sustaining infusion has been calculated on grossly overestimated clearances.

It is well to measure the concentration of materials in the sustaining infusion by the same methods utilized for the plasma since some preparations of inulin and p-aminohippurate are not pure.

Although a 4 hr equilibrium period is the more conservative, infusion equilibrium with inulin was achieved within 1–1$^1/_2$ hr in 45 of 51 observations. If the shorter period is used, changing plasma concentrations will reveal those instances in which infusion equilibrium was not achieved.

The impracticality of measuring Tm_{PAH} because of the large amount of PAH required in the infusion technique refers to the cost of the material. The method can be applied when it is difficult or impossible to obtain accurate urine collection.

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II. Methods of Study of Body Water Compartments (Space Techniques)

NORMAN DEANE, *New York University*

Present methods for study of body water are based on a dilution principle. In the case of a substance restricted to a single compartment, the greatest volume of dilution is, of course, equal to the volume of the compartment. This volume (the volume of distribution) is calculated by dividing the amount of the substance present in the compartment by the concentration of substance per unit of water in any part of the compartment from which a sample may be obtained.

Certain fundamental conditions must be fulfilled in this calculation. (1) The total amount of the substance present in the compartment must be accurately known, so the method should include means of quantitative correction for significant loss through excretion or metabolism. (2) The method should have an internal check which will indicate that in each measurement complete distribution throughout the particular compartment has been attained. (3) The concentration of the substance should be the same throughout the compartment at the time of sampling. The nature of the substance administered and the physiologic state of the subject will determine the procedures to be used if these conditions are to be met.

I. EXTRACELLULAR FLUID

The volumes of distribution of inulin and sucrose more closely approximate the extracellular fluid volume in man than do the volumes of distribution of test substances formerly used (5, 14). They are biologically inert in man and can be quantitatively recovered in the urine after intravenous injection. Because of rapid excretion, true plasma-interstitial fluid equilibrium does not occur after a single intravenous injection (28), so that continuous infusion techniques have been devised to insure complete distribution. These substances may be used in either of the methods described here.

1. *CONSTANT INFUSION-URINE COLLECTION TECHNIQUE*

This method (13, 32) for measurement of extracellular fluid in dog and man involves the use of a priming injection of inulin and an approximately constant rate of infusion to maintain a constant plasma concentration during the interval required for inulin to attain uniform distribution throughout the extracellular fluid. The duration of the infusion, in the absence of increased extracellular fluid, is 2 hr in the dog and 5 hr in man. At the end of this time a plasma sample is obtained, the bladder emptied and the infusion discontinued. The urine is collected for 5 hr in the dog and 12 hr in man. The inulin volume of distribution is equal to the amount of inulin recovered in the urine (after correction for urine inulinoid blank) divided by the concentration of inulin in plasma water.

a) *Procedure.*—1) The subject is hydrated so that the urine flow is 2–4 cc/min while the sample for the rate of excretion of inulinoid blank is collected and also when the final bladder wash-out is performed.*

2) The subject is catheterized with a multiple-eyed catheter and a timed urine sample is collected for determination of the rate of excretion urine blank (U_0V).

3) A control blood sample is obtained for determination of plasma blank concentration (B_0).

4) The inulin priming injection is administered. The priming injection is calculated assuming an inulin volume of distribution of 18 per cent of body weight and an intended plasma concentration of 20 mg/100 cc.

$$\text{Inulin priming injection (mg)} = [\text{wgt of subject (kg)}][0.18][0.2][1{,}000]$$

5) The inulin sustaining infusion is started after the priming injection is completed. An infusion rate of 0.5–1.0 cc/min is preferred. Concentration of inulin in the sustaining infusion is calculated on the basis of infusion rate, intended plasma concentration (20 mg/100 cc) and estimated rate of glomerular filtration.

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* The technique of bladder wash-out is described by Selkurt in Volume 1 of *Methods in Medical Research*, page 191.

$$\text{Inulin concen. in sustaining infusion (mg/cc)} = \frac{\begin{bmatrix}\text{estimated filtration}\\ \text{rate (cc/min)}\end{bmatrix}\begin{bmatrix}\text{intended plasma}\\ \text{concen. (mg/cc)}\end{bmatrix}}{[\text{infusion rate (cc/min)}]}$$

6) The infusion is continued for the required length of time and blood samples taken to check the constancy of the plasma concentration. A final blood sample is obtained before the infusion is discontinued.

7) The bladder is emptied and washed with saline and air.

8) The infusion is stopped on completion of the bladder washout.

9) All urine is collected for the next 12 hr.

b) *Calculation:*

$$\text{Inulin vol of distribution (cc)} = \frac{\begin{bmatrix}\text{total amt of inulin}\\ \text{recovered (mg)}\end{bmatrix} - \begin{bmatrix}\text{rate of excretion}\\ \text{of } U_0V \text{ (mg/min)}\end{bmatrix}\begin{bmatrix}\text{duration of urine}\\ \text{coll. (min)}\end{bmatrix}}{[\text{inulin concen. in plasma water (mg/cc)}]}$$

2. CALIBRATED INFUSION TECHNIQUE

The novel feature in this method of measurement of extracellular fluid in man, using sucrose (5), is the calculation of the virtual volume of distribution at any instant after the start of an extremely constant intravenous infusion without use of a priming injection.

The virtual volume of distribution of any nonmetabolized solute x at any time t is given by the expression

$$V_x = \frac{\Sigma IV - \Sigma UV}{P}$$

where V_x is the virtual volume of distribution (cc), ΣIV the tota quantity of solute that has been infused (mg), ΣUV the tota quantity of solute that has been excreted in the urine (mg), and P the concentration of the substance in the plasma (mg/cc). The value for V_x increases and approaches a maximal volume which is identical with the volume of distribution expected at equilibrium, i.e., when P has been maintained constant for some period of time. With sucrose, that value of V_s which remains constant with prolongation of the infusion is taken as the extracellular fluid volume. An infusion of 3 hr is recommended in the nonedematous adult, although maximal volume of distribution is usually reached in a shorter period.

It is of critical importance in this method that the composition of the infusion be known with great accuracy and that the rate of infusion be kept very constant and be known exactly, because slight errors in IV compound with time in the calculation of ΣIV and, therefore, in all derivative calculations. We have infused a 9.5–10.5 per cent sucrose solution with a Bowman constant infusion pump,† delivering the infusion fluid at a rate of 0.665–0.700 cc/min. When this pump is powered through a Sola constant voltage transformer, the rate of infusion, as checked at 30 and 60 min intervals, remains constant with an accuracy of ±2 per cent. The infusion reservoir consists of a series of 20 and 40 cc glass bulbs with calibrated stems connected by ground-glass joints, permitting the rate of the infusion to be checked from time to time. The volumes delivered by the bulbs are reproducible within ±1 per cent despite repeated autoclaving. Care must be taken to keep them chemically clean so that drainage will be complete. It is essential that a continuous check of this type be kept on the rate of the pump throughout the infusion.

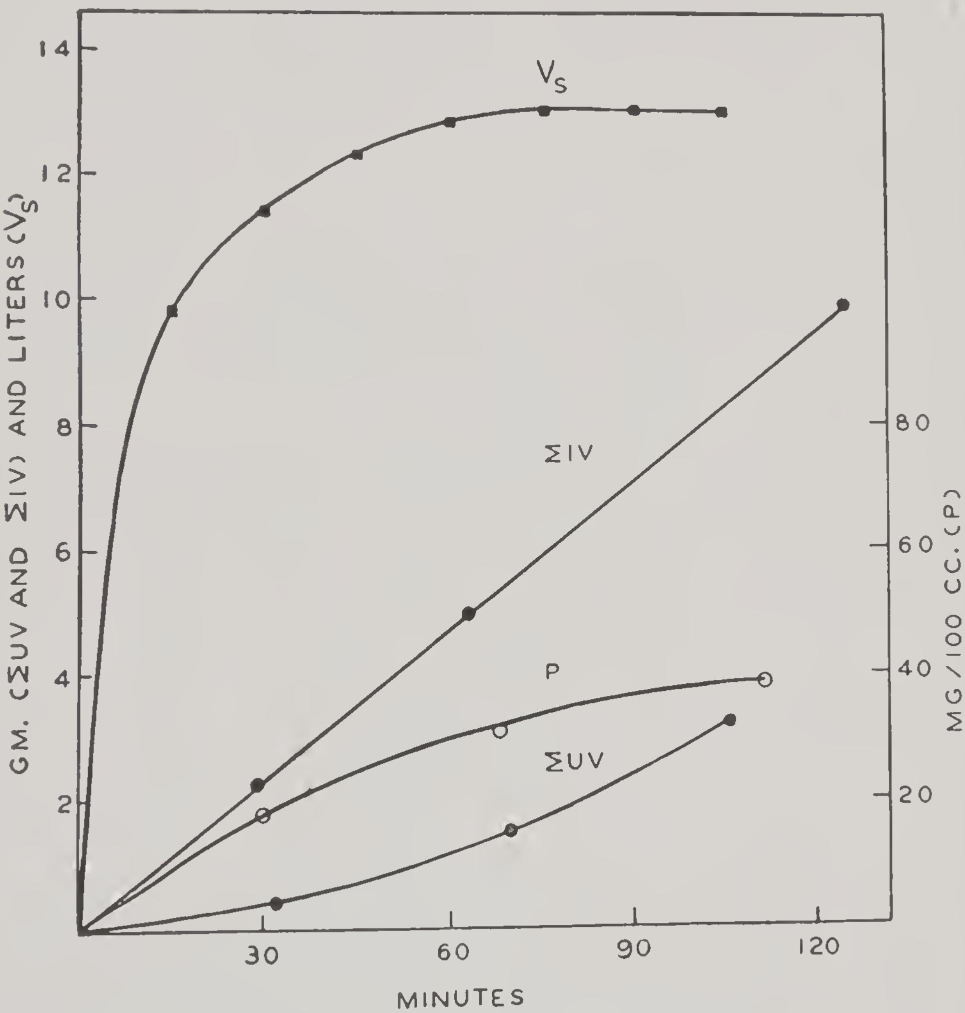

FIG. 1.—Typical experiment showing the virtual volume of distribution of sucrose (V_s) during infusion at a constant rate.

a) Procedure.—1) The subject omits breakfast and is hydrated to produce a urine flow greater than 3 cc/min throughout the infusion.

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† Manufactured by Process and Instruments Corporation, Brooklyn.

2) The subject is catheterized with a multiple-eyed catheter. The bladder is emptied.

3) A control blood sample is obtained for determination of plasma blank concentration, and plastic intravenous tubing is inserted into the vein (18).

4) The exact time is noted and the infusion started through the plastic tubing. The volume infused is obtained by noting the exact time at which the fluid level passes the calibration marks on the infusion apparatus.

5) Timed urine samples are collected at 30 min intervals during the infusion. Thorough saline and air wash-outs of the bladder are used in terminating the collection of each sample.

6) Plasma samples are obtained at 30–40 min intervals during the infusion.

b) Calculation.—The total quantity of sucrose infused (ΣIV) at successive intervals is plotted against elapsed time. These points should fall on a straight line if the rate of infusion has been constant (see ΣIV, Fig. 1). The total amount of sucrose excreted (ΣUV) is calculated for each successive urine sample (see ΣUV, Fig. 1). These points should fall along a smooth curve and serve as a check on the accuracy of urine collection and analysis. The plasma concentration of sucrose, as determined at various intervals, is also plotted against time (P, Fig. 1). The virtual volume of distribution of sucrose (V_s) at any time during the infusion is obtained by inserting the appropriate interpolated values for ΣIV, ΣUV and P in the equation (V_s, Fig. 1).

The term P may be corrected for water content in this operation, but this involves superfluous labor and it is sufficient to make the preliminary calculations in terms of P as analytically observed and to apply the correction for plasma water only to the definitive figure for maximal volume of distribution. This correction is effected by multiplying the final volume of distribution by the fraction of water in the plasma.

At the rates of infusion used here (60.0–80.0 mg/min), the error involved in neglecting blank excretion is less than 4 per cent of ΣUV at 60 min and less than 1 per cent of ΣUV at the end of 120 min. The blank excretion is usually less than 1 per cent of $\Sigma IV - \Sigma UV$ at the end of 60 min. Thus errors in V_s are minimal even if the blank excretion is omitted.

3. ANALYTICAL PROCEDURE

Inulin or sucrose may be determined by Roe's resorcinol method as modified by Schreiner (31). This method depends on the reaction of resorcinol with the fructose liberated by hydrolysis of either substance. Plasma filtrates for inulin analysis are prepared by a cadmium sulfate precipitation (36). For sucrose, the plasma is precipitated by Nelson's (23) modification of the Somogyi zinc sulfate method. Urines are diluted directly unless protein is present, in which case they must be precipitated in the same manner as plasma before dilution.

1) *Preparation of plasma filtrate for inulin analysis.—a*) Reagents.—(1) Cadmium sulfate solution: 34.67 g $3CdSO_4 \cdot 8H_2O$ plus 169 cc 1N H_2SO_4 (exact) made up to 1,000 cc and filtered if necessary. (2) 1.1N NaOH (exact).

b) Procedure.—Two cc of plasma is placed in a 250 cc Erlenmeyer flask. Water, for the appropriate precipitation dilution, is added and mixed. Six cc of the cadmium sulfate solution is added with mixing. Two cc of NaOH is added dropwise with mixing. The flasks are stoppered, shaken well and allowed to stand for 10 min with some additional shaking. The solutions are centrifuged and the supernatant fluid is filtered through washed cotton.

2) *Preparation of plasma filtrate for sucrose analysis.—a*) Reagents.—(1) 5 per cent $ZnSO_4 \cdot 6H_2O$. (2) Approximately 0.3N $Ba(OH)_2$ (store in a bottle protected from CO_2). The $ZnSO_4$ and $Ba(OH)_2$ solutions are adjusted so that 4.7–4.8 cc of $Ba(OH)_2$ solution will produce a definite pink to phenolphthalein with 5 cc of the $ZnSO_4$. The titration is carried out by diluting the $ZnSO_4$ solution to 20 cc with water and adding the $Ba(OH)_2$ with constant mixing.

b) Procedure.—Two cc of plasma is placed in a 125 cc Erlenmeyer flask. Water, for the appropriate precipitation dilution, is added and mixed. Four cc of $Ba(OH)_2$ solution is added slowly with mixing. Four cc of $ZnSO_4$ solution is added slowly with mixing. The flasks are stoppered, shaken well and allowed to stand for 5 min. The solutions are centrifuged and the supernatant fluid is filtered through washed cotton.

3) *Determination of sucrose or inulin in plasma filtrate or urine.—a*) Standards.—A series of standard solutions is run in duplicate with each group of unknown determinations. Recommended concentrations for the standard solutions are 1, 2 and 3 mg/100 cc for inulin and 1, 2, 4 and 6 mg/100 cc for sucrose.

b) Reagents.—(1) 0.1 per cent solution of resorcinol in 95 per cent alcohol (prepared daily). (2) 30 per cent HCl, prepared by adding 22.4 cc of water to 100 cc of concentrated HCl.

c) Procedure.—Two cc of the plasma filtrate, diluted urine or standard solution is pipetted into a 15 × 200 mm Pyrex test tube. Two cc of resorcinol solution is added. Five cc of HCl reagent is added. The tube is shaken and covered with a glass tear. The tubes are placed in a water bath at 80 C (± 1 C) for 25 min and immediately cooled in tap water for a minimum of 3 min.

Color intensity is read in a Coleman Junior spectrophotometer with matched cuvets at a wavelength of 490 mμ using an aqueous reagent blank for 100 per cent transmission. This measurement should be carried out promptly, although the color developed is reasonably stable for over an hour.

Critique of methods.—The calibrated infusion technique is a more satisfactory approach to the measurement of extracellular fluid than the constant infusion-total urine collection method. Absence of the requirement for lengthy and complete urine collection permits application of the method to children and to most adults. If sucrose is used, a valid determination is usually completed within 180 min. That uniform distribution throughout the extracellular fluid has been achieved in each determination is revealed by the attainment of a constant volume of distribution. Evidence that maximal volume of distribution has been achieved in the constant infusion-total urine collection method can only be obtained by repeating the procedure over a longer period in the same subject.

If facilities are not available for administration of an infusion of the requisite constancy, the calibrated infusion technique cannot be used successfully and the constant infusion-total urine collection method should be used. Since it may be anticipated that plasma-interstitial fluid equilibrium is obtained more rapidly with sucrose than with inulin, it is recommended for use in man by either method. Only inulin should be used for measurement of extracellular fluid in dogs, since sucrose is metabolized in this species (26).

Measurement of increased extracellular fluid volume.—With certain modifications, the calibrated infusion technique may be used to measure increased volumes of extracellular fluid (4). A longer period of infusion is required in all such instances.

In subjects with edema but without sufficient accumulation of pleural or peritoneal fluid to permit convenient sampling of these extracellular fluid depots, the infusion should be continued for 8 hr if possible. Results are analyzed in the manner previously described. Samples of edema fluid should be collected, before the infusion is interrupted as additional evidence that plasma-edema fluid equilibrium has been accomplished.

In edematous subjects with accumulations of ascitic and/or pleural fluid, the infusion should be continued as long as possible. The shortest time in which we have noted plasma-ascitic fluid equilibrium has been 8 hr (4). Samples of the pleural, edema and peritoneal fluids should be obtained during the infusion and used to define the time course of sucrose concentration in the extracellular depots and demonstrate equilibrium distribution.

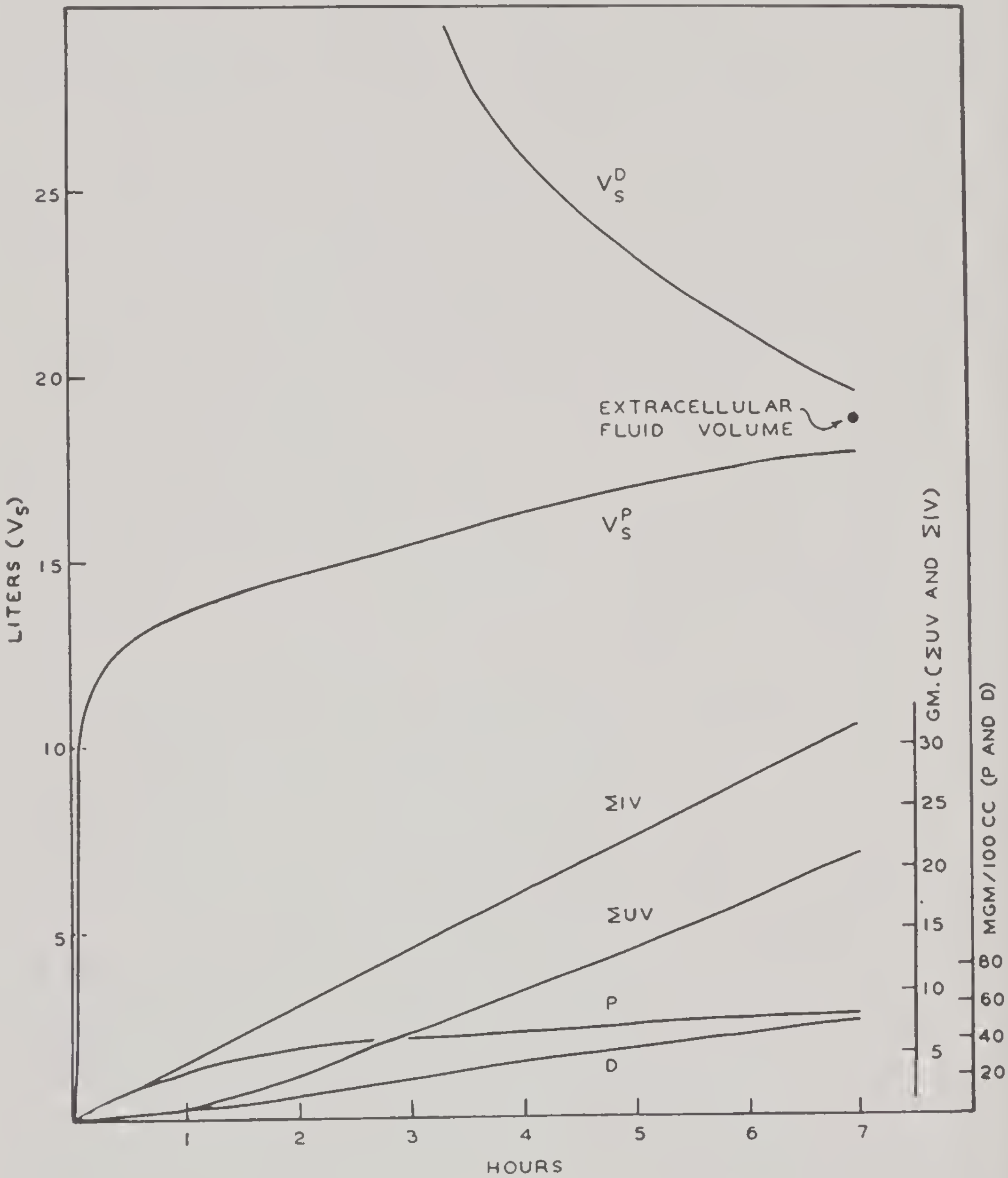

FIG. 2.—Virtual volume of distribution of sucrose as calculated from plasma concentrations (V_s^p) and ascitic fluid concentrations (V_s^d) in a patient with hepatic cirrhosis.

If maximal volume of distribution has not been reached, these concentrations may permit accurate approximation of extracellular fluid volume by application of the following principle. At equilibrium, the extracellular fluid volume may be calculated by dividing the amount of sucrose in the body ($\Sigma IV - \Sigma UV$) by sucrose concentration in plasma water, pleural fluid, ascitic fluid or edema fluid. Before equilibrium distribution, all volumes of distribution are virtual and, when calculated from the plasma concentration, increase to the limiting value of the maximal volume of distribution. However, the virtual volume of distribution, calculated from the concentration in the extracellular fluid depot which is slowest to equilibrate, is initially infinitely large and *decreases* to the volume calculated from the plasma concentration.

The virtual volumes of distribution based on plasma and extracellular depot (pleural, ascitic or edema fluid) concentration are plotted against elapsed time (see V_s^p and V_s^d, Fig. 2). If these estimates are within 10 per cent of one another at the time the infusion is discontinued, the average volume or the volume obtained by extrapolation is a good estimate of extracellular fluid volume, despite the fact that uniform distribution has not been attained.

Interpretation of dilution (space) techniques using radiosodium, radiochloride and bromide.—It is now recognized that sodium and chloride are normally present in certain cells and that estimations of extracellular fluid volume by dilution of these substances are not valid. Indeed, the use of volume units and the term "space" in reporting such dilution studies tends to obscure the important information which their use should disclose.

The fundamental concepts of tracer theory require that isotopic Na and Cl be regarded as diluted in body Na and Cl and not in body water. In reality, the measurement is that of a *mass* and not a "space." It is assumed that when the isotope has reached maximal dilution the ratio of the concentration of isotope (counts/min) to that of the nonisotopic form (mEq)[‡] is the same in all tissues of the body regardless of water content.

After corrections for radioactive decay, the total body electrolyte may be calculated as

$$\begin{matrix}\text{Total body}\\ \text{electrolyte}\\ \text{(mEq)}\end{matrix} = \frac{\left[\begin{matrix}\text{amt of isotope in-}\\ \text{jected (counts/min)}\end{matrix}\right] - \left[\begin{matrix}\text{amt of isotope ex-}\\ \text{creted (counts/min)}\end{matrix}\right]}{\left[\dfrac{\text{isotope concen. in plasma (counts/min/cc)}}{\text{electrolyte concen. in plasma (mEq/cc)}}\right]}$$

As may be seen from the formula, correction must be made for urinary excretion of the isotope during the time of complete distribution.

It has been demonstrated that distribution of administered bromide is similar to that of chloride in tissues and body fluids except for brain and cerebrospinal fluid (39, 41). Consequently, total body chloride may be estimated by bromide dilution (8, 40). When complete distribution of bromide is effected, the calculation is

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[‡] This is termed the specific activity (counts/min/mEq).

$$\text{Total body Cl (mEq)} = \frac{\left[\begin{array}{c}\text{amt of Br}^- \text{ in-}\\ \text{jected (mg)}\end{array}\right] - \left[\begin{array}{c}\text{amt of Br}^- \text{ ex-}\\ \text{creted (mg)}\end{array}\right]}{\left[\dfrac{\text{Br}^- \text{ concn. in plasma (mg/cc)}}{\text{Cl concn. in plasma (mEq/cc)}}\right]}$$

Simultaneous measurements of total body electrolyte (Na or Cl) and body water distribution permit calculation of intracellular electrolyte and intracellular electrolyte concentration. This principle has been applied to dogs (19) and man (6, 7).

It is not possible at present to state with certainty the extent to which so-called "bound" electrolyte exists in man. "Bound" electrolyte, presumably in bone and possibly in other tissues, would not be measured by these techniques. In the absence of definitive information on this point, the term "exchangeable" (2) has been proposed as descriptive of the physiologic state of the total electrolyte mass as measured by the isotope dilution technique. It seems appropriate, then, to refer to the values obtained as total exchangeable Na or Cl, etc.

A minimal equilibration time of 24 hr for sodium, bromide and chloride is recommended for the most accurate measurements.

B. TOTAL BODY WATER

1. *Isotope techniques.*—The use of deuterium oxide (D_2O) for measurement of total body water in man was initiated by Hevesy and Hofer (15). Pace and his co-workers (25) have described the use of tritiated water (water labeled with tritium [HTO], the radioactive isotope of hydrogen, with mass 3) for this measurement. On theoretical grounds these substances seem to be ideal. In tracer quantities, the biologic behavior of "tagged" water is not significantly different from that of the nonisotopic form. The greatest volume of dilution has been shown to agree closely with measurements of total body water obtained by desiccation (21, 22, 25).

PROCEDURE

1) The substance is administered by a single injection with a calibrated syringe or buret. From 50 to 100 cc of D_2O is the usual dose; larger amounts are required if total body water is much increased. In general, the precision of the method improves with the use of larger amounts, since the higher equilibrium D_2O concentration will be in a range of increased analytic accuracy. The range of equilibrium concentration with these amounts will be from 0.1 to 0.2 per cent D_2O. The dose of tritiated water may range from 5.0 to 25.0 mc, which yields equilibrium concentrations of 0.1–0.5 mc/l. If serial studies are being conducted, a blood sample should

be obtained before injection for determination of deuterium or tritium blank.

2) In the absence of abnormal increments of body water, equilibrium distribution will be attained in 2 hr. At least 2 blood samples should be collected between the 2d and 4th hours to demonstrate that equilibrium has been reached. With increased total body water and abnormal extracellular depots, an interval of 4–6 hr should elapse before the 1st sample is obtained and additional blood specimens should be collected to the 12th hour. In such instances samples of ascitic, pleural or edema fluid should be obtained at the time of collection of the last blood sample as additional demonstration that homogeneous distribution has been attained.

3) If it is anticipated that the time required for equilibration will be in excess of 4 hr, all urine should be collected from the time of injection. Otherwise it is sufficient to estimate the loss during the first 2 hr through all routes as 0.4 per cent of the amount injected (29).

Analysis of deuterium and tritium.—Limitations of space prevent a complete description of the methodology pertaining to deuterium and tritium analyses.

Deuterium concentration may be determined directly, after conversion of the sample to a gas, in the mass spectrometer (for a general description of this instrument, see (20)). Detailed information concerning types of instruments and applications of mass spectrometric methods has been presented by Siri (34) and Nier (24), and Solomon, Edelman and Soloway (38) have presented specific considerations of the mass spectrometric analysis of deuterium in body fluids.

The concentration of D_2O in water may be determined indirectly by measuring certain physical properties of deuterium oxide-water mixtures which vary with concentration of the former. Robertson (27) has described the indirect methods for measuring D_2O with particular reference to methods based on density differences (falling-drop, free-float, pycnometer and diffusion gradient methods) and changes in the refractive index. Of these methods, the falling-drop method using o-fluorotoluene as initiated by Keston, Rittenberg and Schoenheimer (17) has been widely used in biologic investigations.

The fact that tritium is radioactive permits determination of emitted radiation. Tritium is a β-emitter with a half-life of 10.7 yr. The radiation is of extremely low energy (0.011 mev), so that special techniques must be applied. Pace and his co-workers (25) have devised a counting assembly which involves the introduction of HTO as water vapor at low pressure into a Geiger tube, the emission being counted directly. However, the sample may be converted to the gas phase before introduction into the tube. Eidinoff and Knoll (10) have compared gas counting with a method for measuring tritium in solid samples. The vibrating reed electrometer has also been adapted for tritium analysis (35).

Calculation.—Total body water is calculated by dividing the total amount of "tagged" water administered (after correction for urinary excretion) by the equilibrium concentration in plasma water.

2. *Antipyrine method.*—The use of antipyrine in measurement of total body water, proposed by Soberman and co-workers (37), has been described elsewhere in this series (1), as has also the use of N-acetyl 4-amino antipyrine for this purpose (1). Descriptions of these methods are therefore omitted here.

Critique of methods.—Except for the error introduced by the exchange of the isotope with hydrogen atoms of organic molecules, D_2O and HTO are ideal substances for the measurement of total body water because of their rapid equilibration and successful biologic masquerade as water. For D_2O, the error through exchange has been estimated to produce results which are high by a factor of 1.0–1.5 per cent of body weight (700–1,000 cc) (38). Unsatisfactory studies with these materials can only result if insufficient time is allowed for homogeneous distribution or if there are technical errors in the administration of the infusion or collection and analysis of the blood samples. The methods have the disadvantage that the analytical techniques are complex and require special equipment and trained personnel. It should be pointed out that there is a fairly considerable change, percentagewise, in the molecular weight of these substances as compared to water. The ratio of the molecular weights of D_2O and HTO to water is 1.11. Hence there is a possibility that their rates of internal exchange in the body may differ from those of nonisotopic water.

The antipyrine method is a valuable contribution to methodology in this field because of the simplicity of analytical procedure and the fact that multiple studies are economically feasible. It is important that the procedure be modified if total body water is obviously increased. The 1st blood sample should not be taken before 6–8 hr and the additional samples should be obtained for as long as possible, or a minimum of 12 hr. If there is a pronounced increase in extracellular fluid volume with ascites, edema and pleural effusion, samples should be collected from these sites at intervals after the 6th hour. This is essential for 2 reasons. First, it aids in estimating the time at which homogeneous distribution was achieved. No prior plasma concentration is valid for extrapolation to zero time.

Theoretically, the lack of equilibration should be revealed by a changing slope of the semilogarithmic plot; actually, the rate of equilibration in certain slowly exchanging depots may be comparable to the rate of metabolism, in which case the graph will not separate these components.

The 2d reason derives from the fact that, for a valid measurement, the antipyrine concentration must demonstrably be the same in all parts of extracellular fluid. If differences of concentration or slope occur among components of extracellular fluid, this condition has not been fulfilled. This point requires emphasis because in some subjects with conspicuously increased extracellular fluid the concentration of antipyrine in pleural or ascitic fluid may exceed that in the plasma water, a discrepancy that may persist despite a subsequent decrease in both plasma and extracellular depot concentrations as a result of metabolism (4). The error leads to an overestimate of total body water.

C. INTRACELLULAR WATER

The only method available for the estimation of intracellular water is indirect; i.e., intracellular water must be calculated as the difference between total body water and extracellular fluid. The methods for measuring these 2 compartments have been described in detail. Obviously the accuracy of the derived value for intracellular water will be determined by the individual errors in the primary methods.

Normal and abnormal patterns of body water distribution can be effectively expressed if intracellular water is calculated as per cent of total body water and also per cent of cellular mass (body weight minus weight of the extracellular fluid) as well as per cent of body weight. In 11 normal males, intracellular water averaged 70.2 per cent of body water (range 64.9–74.5), 50.3 per cent of cellular mass (41.8–59.6) and 40.6 per cent of body weight (range 34.7–48.0) (3). Changes in these terms readily show shifts of fluid and alterations in cellular hydration (intracellular edema or dehydration).

Comment by David P. Earle, Jr.

Although time-consuming, the methods described here represent considerable improvements in the measurement of body fluid compartments. They are far superior to methods based on chloride balance, distribution of thiocyanate, and the like. However, extreme care must be devoted to all technical details if accurate results are to be obtained. Further, it must be kept in mind that any "space" measured by the distribution of inulin, sucrose and antipyrine is translated to extracellular fluid or total body water by inference. Nevertheless, these methods are the best available.

The calibrated infusion technique for the measurement of extracellular fluid is particularly attractive because of the short time required and because of the internal check available when a constant volume of distribution is achieved. However, if uniform distribution of sucrose in edematous subjects is not achieved in all parts of extracellular fluid (plasma, interstitial, pleural or ascitic fluid) at the same rate, the interpretation of results is open to question. The alternative method of measuring sucrose in all available fluids is tedious. In the case of inulin, a constant infusion of as long as 24 hr or more may be required before the concentration of inulin in pleural or ascitic fluid becomes equal to that in plasma.

When the resorcinol method, which does not involve the use of yeast to destroy glucose or fructose in the blood, is used to measure inulin or sucrose it is essential that the subject be in the fasting state. The edematous subject may therefore be required to go without food for almost 24 hr in view of the long time required to achieve uniform distribution.

Comment by Isidore S. Edelman

Dr. Deane is to be congratulated on covering the various techniques in such concise and useful form. The following remarks may be pertinent.

1. There is some evidence that thiosulfate may prove to be as accurate and more convenient in measurement of extracellular fluid space than either inulin or sucrose.

2. It is true, as Moore (30) pointed out, that the exchangeable sodium (mEq) is the important parameter measurable with radiosodium. Nevertheless, the sodium space (liters) may still be a useful and perhaps even a physiologically important value.

3. In the estimate of exchangeable sodium, some 600–900 mEq is contributed by bone sodium (12, 16). Since the penetration of substances for measurement of extracellular fluid into bone has not been reported, we do not know how much of this sodium is included in estimates of intracellular sodium by isotopic dilution methods.

4. A revised and accurate technique for measuring D_2O concentrations by the falling-drop method has been described (30).

5. In low concentrations, as is the case when D_2O is used as a water tracer, almost all of the tracer exists as HDO. The molecular ratio to water being 1.05, the likelihood of a "mass effect" is small.

6. D_2O has been applied to studies on the rates of water exchange between cells and its extracellular environment (9). Present data indicate that 25 per cent of extracellular water is exchanged per minute. These data are obtained by analysis of the pre-equilibrium portion of the arterial D_2O disappearance curve. We do not believe that Flexner's (11) interpretation of the arterial curve in terms of transcapillary water exchange is correct.

Comment by Dr. Deane

1. Use of thiosulfate as a measure of extracellular fluid volume has also been described by Schwartz (33); calculation of volume was made without urine collection from the total plasma clearance during infusion and the

postinfusion decrement in plasma concentration. A rough calculation indicates that the rates of diffusion of sucrose and thiosulfate would be about the same; the comparative advantage of either would presumably depend on the ease and accuracy of chemical methods.

2. The comment that the concept of "sodium space (liters) may still be a useful and perhaps even a physiologically important value" does not accord with Dr. Edelman's recognition and acceptance of the fact that the function measured is exchangeable sodium (mEq).

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III. Electrolyte Excretion Studies in the Dog

LAURENCE G. WESSON, JR., *New York University-Bellevue Medical Center*

The techniques described here are aimed at evaluating the relative contributions to renal electrolyte and water excretion of plasma composition, glomerular filtration rate and tubular function. To this end it is essential that composition of the plasma be maintained at constant controllable levels. Two situations specifically described which illustrate these principles are the effects of expansion of extracellular fluid (p. 181) and urea diuresis (p. 188).

GENERAL PROCEDURES

All experiments are performed on postabsorptive female mongrel dogs accustomed to the procedures. No food is given them for 18 hr. We allow water ad lib but avoid forced hydration the night before an experiment because, in man at least (6), it leaves trace effects on the responses of sodium and water excretion to intravenous saline infusion 12 hr later.

Glomerular filtration rate is measured as the creatinine clearance by the technique of urine collection, and simultaneous plasma determinations* which permits estimates of rapid changes in glomerular filtration rate, as other methods do not (12). Effective renal plasma flow is measured by the clearance of p-aminohippurate at low plasma levels (12).

Sodium and potassium are measured by the flame photometer against an internal lithium standard (3). Chloride is determined by the method of Sendroy as modified by Van Slyke and Hiller (14). Plasma and urine carbonate are computed (9) from total CO_2 and pH, using the Henderson-Hasselbach equation.

Plasma proteins are determined by the copper sulfate method of Van Slyke *et al.* (15). For the dog we use the factor (369) and specific gravity of plasma water (1.0065) described for man, although true dog values are probably somewhat different. When plasma sodium and urea concentration are greatly altered, correction factors are used. The general equation we use is

$$P = 369(S - 1.0065 - K_1 - K_2)$$

where P is plasma protein concentration in g/100 ml, S is measured specific gravity of plasma, K_1 is $0.000253 \times P$ *urea* (mg/ml), and K_2 is 0.0359 (PNa [mEq/ml] − 0.150). Appropriate values of K_n

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* For urine flows less than 0.5 ml/min the bladder is rinsed 3 times with 10 cc of distilled water. For urine flows in excess of 3 ml/min the bladder is not rinsed. Since the renal pelvis empties intermittently, the lower the urine flow, the longer should be the collection periods.

should be substituted in experiments involving other solutes.

The freezing point of plasma and of urine is measured with a thermistor in a Johlin freezing point apparatus (5). The freezing point of biologic fluids can thus be measured as accurately as with a Beckman thermometer but more rapidly and with smaller samples.

THERMISTOR.—A thermistor (Western Electric type 14B) consists of a small bead of copper oxides which displays in the region of 0 C an *increase* in resistance of 250–350 ohm/degree *decrease* in temperature. It is mounted about $^1/_{16}$ in. from the tip of a long medicinal applicator stick. Fine, bare copper wires are soldered to the terminals of the thermistor and run up the stick, where they are soldered to heavier wire leads. This assembly is given 5 coats of waterproof cement, each coat being oven-dried at 100 C for at least 24 hr and the whole assembly, beginning 1 cm above the thermistor bead, is given a single tight wrapping of electrical Scotch tape. The thermistor assembly is incorporated into a standard Wheatstone bridge circuit, (e.g, Leeds and Northrup no. 4760). A 1.5 v dry cell battery serves as current source. Balance is measured with a galvanometer of medium sensitivity (0.01 μ amp/mm). A 1,000 ohm shunt with a snap switch is placed across the poles of the galvanometer for coarse adjustment.

FREEZING POINT DETERMINATION.—The freezing apparatus and procedure are similar to those described by Johlin (5). Samples, 0.5–2.0 ml, are placed in long plastic tubes about 1 cm in diameter (our tubes are made by cementing together portions of shorter tubes). After precooling in a cracked ice bath, a sample tube is placed in the freezing apparatus and the thermistor assembly placed in the tube. The temperature of the freezing mixture is adjusted to about 2° C below the expected freezing point of the sample and the sample cooled to about 0.5° C below its expected freezing point. At this temperature, about 1 per cent of the water is converted to ice on abolition of supercooling. With the galvanometer shunt in the circuit, and with continuous stirring by a gentle up-down motion of the thermistor assembly, the progress of cooling may be followed by the increasing resistance necessary to maintain a null reading on the galvanometer. For best results, there should be continuous current flow, rather than make-break, when the region of the freezing point is reached. Supercooling is abolished with a frosted, 2 mm section of heavy gauge copper wire. This is marked by a rapid decrease in resistance. A plateau is reached in a few seconds and held for several minutes. As soon as the plateau is reached, the galvanometer shunt is opened and the final adjustment of the resistance made. For practical purposes, the resistance of this plateau, read to the nearest 0.5 ohm, may be taken as measuring the freezing point. Thermal equilibrium is reached very quickly.

Actual freezing point is obtained from a standard curve prepared by plotting the freezing point of a graded series of standard solutions (e.g., urea, glucose, NaCl) against their plateau resistances. In practice, we take the average of 3 separate determinations, which should not differ, one from another, by more than 2 ohms. In contrast to the Beckman thermometer, the thermistor resistance element is a continuous source of heat. A gradient of heat flow is established between thermistor and the freezing bath so that the measured temperature is the warmer end of a system in dynamic equilibrium. Accuracy depends primarily on careful temperature control and duplication of conditions between standard and unknown solutions. With practice, the freezing point of 4 samples/-hr (3 determinations/sample) can be analyzed with an accuracy of 0.003° C. The following additional notes on use of this procedure should be useful. The standard curve shifts within a narrow range and should be checked every few hours. We believe that shifting of the standard curve is due largely, if not entirely, to changes in room temperature and humidity. The thermistor assembly should be thoroughly dried when not in use; water penetration with short-circuiting has been our major source of trouble. The battery should be checked every 2 weeks; a weakening battery gives poor checks on duplicate determinations.

Because of the very low heat content of the system compared with the sample (a major factor in permitting ease and accuracy of freezing point measurement), we believe this apparatus can be easily modified to measure the freezing point of samples of 0.1 cc or less.

$$\text{osmolarity} = \frac{\text{freezing point depression of sample}}{(-)1.86^\circ\text{ C}}$$

Osmotic pressure of plasma and urine may be estimated from their composition provided all constituents are known. In certain circumstances a few known substances comprise most of the solute and this value can be approximated. Thus, under urea loading, 98–99 per cent of the solute may consist of urea plus salts of sodium and potassium. Approximately 95 per cent of the osmotically active solute of plasma is salts of sodium and potassium plus urea and glucose. In urea diuresis we use the general formula

$$\text{osmolarity} = [\text{urea}] + [\text{glucose}] + 2([\text{Na}] + [\text{K}]) \times F$$

where F represents the deviation of electrolytes from the freezing point caused by electolytes in contrast to substances such as urea and glucose. For practical purposes, total electrolyte may be considered similar to NaCl, the effect of which on freezing point at different concentrations is obtainable from standard texts, e.g., *International Critical Tables.* F varies in a nonlinear manner from 1.0 at zero electrolyte concentration to 0.90 at 500 mEq/1.

Calculations.—The rate of reabsorption of a substance from the glomerular filtrate is given by the general equation

$$T = (C_F \times P) - (U \times V)$$

where T is the rate of reabsorption (mM/min), C_F is glomerular filtration rate (cc/min), P is plasma concentration of the substance, and $C_F \times P$ is the filtered load (mM/min). U is concentration in the urine (mM/ml), V the urine flow (cc/min), and $U \times V$ is the urinary excretion rate (mM/min).

The minute excretion rate of an ion being simply computed ($U \times V$), computation of T depends on the values obtained for filtered load ($C_F \times P$). Since the glomerular filtrate has essentially the composition of a simple plasma ultrafiltrate, 2 corrections must be applied to the term $C_F \times P$; one is for the fraction, w, of filtrable volume in the plasma, and the other, k, for the inequalities of distribution of ions produced by restriction to the vascular compartment of the negatively charged protein molecules (Donnan effect). No significant inaccuracy is involved in the assumption that the *weight* of plasma proteins is a reasonably close measure of the *volume* of nonfiltrable plasma materials, so that $w = P_{prot}$ (g/cc). Donnan factors are obtained from the literature (2, 4) by plotting plasma protein concentration against corresponding ratios of extravascular over vascular ion concentrations as measured per kilogram of water. The best straight line is then drawn through the point of protein concentration = 0 and ion ratio = 1. The data do not establish the relationship as truly linear and the estimates can probably be refined. For a protein concentration difference of 6 g per cent between plasma and ultrafiltrate, we have computed k values of 1.03 for Cl^- and HCO_3^-, 0.95 for Na^+ and 0.89 for K^+. The rate of formation of glomerular filtrate is now wC_F, and the concentration of any ion in the filtrate is kP/w. The tubular load, after cancellation of terms, is $C_F \times kP$.

EXTRACELLULAR FLUID VOLUME†

It has long been recognized that the kidney plays a major role in regulation of the *composition* of the blood plasma and extracellular fluid, and many of the mechanisms involved have been elucidated.

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† For a review of the literature of this field, see (13).

The kidney's role in regulating the *volume* of the extracellular fluid is less clear and the mechanisms involved are obscure.

In man, when extracellular fluid volume is acutely expanded, as by injection of Locke's solution, excretion of salt and water increases in a few minutes and reaches a maximum in a few hours. The urine may attain a flow of 5–10 cc/min and the inulin U/P ratio may fall in the range 15–20. If the extracellular fluid volume of a dog is acutely expanded, increase of excretion of salt and water begins more rapidly than in man and reaches a maximum within an hour, with creatinine U/P ratios of 5–10. High, complexly varying rates of salt and water excretion continue until the amounts eliminated equal or slightly exceed those injected. Concomitant effects of loading are increases in central venous pressure and dilution of plasma proteins; the former does not prevent the increase in salt output, nor does the latter account for the increase in filtration rate observed in the dog.

In contrast, simple depletion of extracellular fluid volume in man or dog depresses outputs of salt and water, so that the urine may contain no more than traces of sodium, while water loads are slowly excreted. In this circumstance the body tends to restore extracellular fluid volume by diluting that which remains with water, so that plasma sodium concentration is commonly decreased. In spite of the fact that the plasma becomes hypotonic, water diuresis fails.

These responses to repletion and depletion of extracellular fluid pose 2 problems: (1) what are the changes in renal function which result in retention or excretion of salt and water, and (2) what organ or tissue responds to changes in extracellular fluid volume and signals for an appropriate adjustment of renal function?

Changes in renal excretion of salt result from changes either in filtered load or in tubular reabsorptive function. In the dog, filtered load, as influenced by changes in filtration rate, seems to be the more important control of salt excretion. Administration of Locke's solution or feeding of large amounts of NaCl may increase filtration rate by as much as 50 or 75 per cent. Almost certainly as a result of this, there occurs a massive increase in excretion of salt and water, so that saline equal in quantity to as much as half the normal extracellular fluid volume may be excreted over 2–4 hr; filtration rate returns to the control level as elimination of the load becomes complete. Participation of changes in tubular reabsorption in this response has not been fully evaluated. During acute loading, the amount of salt reabsorbed increases, whereas it might have been expected to decrease; however, this increase in reabsorption is more than compensated for by the increase in filtration. Whether or not a decrease in reabsorption ensues when the loading is sustained for more than 24 hr is not known.

Depletion of the extracellular fluid in the dog causes a drop in filtration rate sufficient to account for the ensuing retention of salt water; whether or not tubular reabsorption is also increased is not known. Thus, in the dog, changes in glomerular filtration rate are probably the primary mechanism in regulating extracellular fluid volume.

Man does not exhibit a comparable flexibility of filtration rate. The filtration rate of a human subject responds to a load of Locke's solution by no change or by increases of only 10–15 per cent.[‡] At the same time, excretion of a load of Locke's solution is much more sluggish and may require 24–28 hr. Although man is unable to draw freely on filtration rate in control of salt excretion, tubular reabsorptive capacity under conditions of saline loading or a high salt diet is believed to change in the direction of a slight but definite decrease, so that salt excretion increases. Salt depletion is accompanied by a decrease in filtration rate which is not as pronounced as in the dog. The extent to which tubular reabsorptive capacity may increase in salt depletion in man is not clear.

The end-organs responsive to change in extracellular fluid volume and the pathways by which they evoke the appropriate changes in renal function are unknown. Some scant evidence suggests that the volume-sensitive organ is at least partially integrated with the osmoreceptor apparatus of the neurohypophysis. Thus, Ladd (6) has shown that water diuresis conspicuously alters the kidney's response to a saline load infused some hours later, so that both salt and water excretions are enhanced. Nelson and Welt (8) have adduced evidence that water retention and creation of hypotonic plasma in cirrhotics may be due to enhanced antidiuretic hormone production.

The procedure that follows was devised to study renal function under conditions such that volume and composition of the expanded extracellular fluid are constant during the experiment. Ideally, we should like to fix all conditions and examine the results of controlled, independent variations in each of the factors involved in salt excretion. This has not proved possible in the intact dog since no practical method exists which will stabilize filtration rate. Nevertheless, the problems can be simplified by careful regulation of variables subject to control by the investigator.

[‡] In certain circumstances, filtration rate in man may show large changes which probably contribute extensively to simultaneous changes in salt excretion. Infusion of hypertonic NaCl sometimes as much as doubles filtration rate: a sharp rise in filtration rate usually accompanies the diuresis of nephrotic edema.

EXTRACELLULAR FLUID EXPANSION: EXPERIMENTAL PROTOCOL

The object of this experiment was examination of the effects on renal function of an increase in body salt and water without change in composition.

a) Solutions.—A: Creatinine (Cr) and PAH prime. Per kilogram of body weight: Cr, 50 mg; PAH, 8 mg; Locke's solution (same as saline prime, C below), 1.5 ml. The prime will produce a plasma Cr concentration of about 12 and PAH concentration of about 2.0 mg per cent.

B: Cr and PAH sustaining solution. Composition is computed from estimated excretion rate ($P \times C$, the latter term determined by experience) divided by pump infusion rate. Rate of infusion should be low, less than 1.0 cc/min, to avoid introducing more fluid than necessary by this route. Cr and PAH may be dissolved in distilled water or saline, as desired; since both are buffers, solutions containing them should be titrated to pH 7.4 with strong acid or base.

C: Saline prime. We use the following formula based on mean calculated plasma ultrafiltrate composition of our animals. NaCl, 124 mEq/l; KCl, 5 mEq/l; $NaHCO_3$, 26 mEq/l; $CaCl_2$, 1 mEq/l; glucose, 100 mg/100 cc; creatinine, 12 mg/100 cc; PAH, 2.0 mg/100 cc. The solutions, so far as possible, should be prepared with pyrogen-free water, and all glassware and tubing should be chemically clean.

D: Saline solution. This is a series of solutions designed to approximate closely the electrolyte composition of the copious urines which may be excreted during an experiment; the appropriate solution is infused into the animal in a volume equal to the urine flow. The composition of the urine may be computed by rapid analysis during an experiment or predicted from analysis in preliminary experiments.

b) Technique.—The animal is placed on a comfortable dog board. An indwelling plastic catheter (7) about 5 in. long is inserted into each external jugular vein through a thin-walled 14 gauge needle. The needle is withdrawn and an adapter consisting of a truncated 19 or 20 gauge needle is forced into the catheter lumen. The catheters are flushed with a drop of heparin solution (Liquaemin, Hoffmann-La Roche), stoppered by any convenient means and fastened in place with a bit of adhesive and surgical skin clips or by a stitch through the skin. One catheter is for injection of solutions, especially those containing creatinine and PAH; the other is for withdrawing blood samples.

A control blood sample is collected, using 2 syringes. The 1st

0.5 cc of blood drawn is discarded and the sample collected into a syringe containing 0.1 cc of heparin solution for each 10 cc of blood drawn. The catheter is then flushed with a drop of heparin and stoppered. The blood sample is centrifuged at once. If CO_2 and pH are to be measured, the syringe should be oiled and should contain a drop of Hg, the blood delivered under oil through a long needle and centrifuged not more than 5 min. The plasma is then pipetted off (directly into Van Slyke-Neill pipets for CO_2 and pH); the red cells are suspended in an equal volume of saline, filtered through a loose, washed cotton pledget and injected into the animal so that red cell depletion does not complicate the experiment.

The Cr-PAH prime (solution A) is injected, followed by infusion of the sustaining mixture (solution B). Thirty min is allowed for attainment of sufficient equilibrium to permit accurate clearance measurements. A constant rate of sustaining infusion is seldom secured by a drip, so a constant infusion pump should be used.§

Collect control clearance periods. The bladder should be washed thoroughly at the beginning with 3 or 4 vol of distilled water and each tested for completeness of recovery. We collect 3 control periods of 20 min each.

The number and timing of collection of blood samples should be arranged to yield the most accurate possible curve of plasma concentrations; sampling is done at least once and often twice during each urine collection period. The infusion pump is switched off just before and during blood collection so as to avoid cross-contamination between the catheter orifices; the blood should therefore be withdrawn as rapidly as possible.

The saline prime (solution C) may be started during bladder wash-out of the final control period. Volume and rate of administration are predetermined for each experiment. As much as 150 cc/kg has been injected at a rate of 200 cc/min. Rapid infusions are most conveniently delivered through the blood catheter. Infusions at slower rates may be given through a Y-tube at the infusion catheter.

Profound changes in renal function and urine flow and composition result from body fluid expansion. Their magnitude and velocity are related to the size and rate of infusion of the saline prime. Urinary outputs of water and electrolyte increase greatly and composition of the urine changes continually. Urinary losses would result in loss of the original saline prime and also in temporary but large alterations in plasma composition unless the urinary losses were restored by appropriate infusion. As indicated, we have used 2 methods interchangeably in selecting the infusion, viz., direct rapid analysis and estimation of probable composition. For direct analysis, the urine is analyzed rapidly for Na^+, K^+ and pH[‖] as soon as the clearance period ends. From pH (assuming a normal pCO_2), HCO_3^- is estimated. The balance of the anion to match the sum of Na^+ and K^+ is assumed to be the chloride. A replacement solution equal in volume to the urine collected may be prepared in 30 min by this method. The method of estimation of probable composition follows from the observation that for any given animal and protocol, the urine composition changes according to a definite pattern and time sequence. Consequently, "synthetic urines" which will not differ greatly from those subsequently obtained may be prepared in advance of the experiment. Their use, volume for volume, to replace the succession of urine volumes collected prevents more than minor fluctuations in plasma composition. All saline replacements are administered by drip through a Y-tube in the infusion catheter.

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§ A Bowman infusion pump, model 2 (Process and Instruments Co., Brooklyn) is quite flexible and versatile. For precise work, however, delivery rate must be checked at intervals, as it must be modified to secure a continuous instead of discontinuous range of delivery rates.

OSMOTIC DIURESIS*

Osmotic diuresis, in the broadest sense, may be defined as an increase in urine flow caused by increased rate of solute delivery into the urine. An increased rate of urinary solute excretion may be produced by: (1) Nonionized substances delivered essentially quantitatively into the urine as filtered, e.g., mannitol, sucrose. (2) Nonionized filtered substances whose rate of delivery into the urine is less than that filtered because of losses from the tubule through passive back diffusion (urea). (3) Nonionized substances filtered in excess of the tubular reabsorptive capacity, e.g., glucose. (4) Nonionized substances excreted because of toxic depression of normal tubular reabsorption (phlorizhinized glucose). (5) Ionized substances delivered almost or entirely quantitatively into the urine as filtered, e.g., sulfate, thiosulfate, ferricyanide. (6) Ionized substances filtered in excess of tubular reabsorptive capacity, e.g., bicarbonate, isotonic or hypertonic saline, xanthine diuresis in part. (7) Ionized substances delivered to the urine by tubular secretion, e.g., PAH. (8) Ionized substances which cause toxic depression of normal tubular reabsorption—mercurial diuresis, xanthine diuresis in part.

Conventionally, the term osmotic diuretic applies to types 1–5.

[‖] For pH (and CO_2) determinations, urine is taken by gravity directly into a Van Slyke-Neill pipet from the indwelling bladder catheter at the midpoint of a urine collection period.

* For a review of the literature of this field, see (13).

The particular substance whose presence in the urine is primarily responsible for the increased flow is termed the osmotic or loading agent. The discussion which follows formulates the general basis for a quantitative description of the urine flow during osmotic loading by nonionized substances.

The rate of urine flow is closely correlated with the rate of solute excretion where solute is measured in terms of its osmotic activity (milliosmols/min). From lowest to highest urine flows, a change in solute excretion rate is accompanied by a parallel but not strictly linear change in urine flow. Although urine osmolarity may differ widely from plasma osmolarity at low rates of solute excretion, the variations become less and less as the rate of solute excretion increases. Mathematically, osmolarity of the urine approaches that of the plasma as a limiting value as the rate of solute excretion becomes indefinitely large. This tendency to approach the plasma osmolarity as a limit is observed irrespective of whether the urine tends initially to be hyperosmotic or hyposmotic to the plasma. Expressed in functional terms, small and limited renal concentrating and diluting mechanisms become of less and less relative importance when they are brought to bear on large and increasing quantities of a primary, isosmotic urine.† At lower rates of flow of a primary, isosmotic urine, concentrating and diluting mechanisms may bulk relatively large, so that large deviations from the osmolarity of the plasma may be expected. Implicit in this analysis is a careful distinction between, on one hand, urine osmolarity which approaches equality with the plasma as urinary solute load increases and, on the other hand, activity of concentrating or diluting mechanisms. Activity of the concentrating mechanism is measured as the volume of water reabsorbed/min *without solute* from an otherwise isosmotic urine. Activity of the diluting mechanism is measured as volume of water virtually contributed/min *without solute* to an otherwise isosmotic urine. Obviously, if both mechanisms should be simultaneously operative, we can measure only the difference between them.

Thus the basis is provided for a separation of the urine flow into 2 portions: a primary, isotonic urine, which is secondarily modified by the net operation of concentrating and diluting mechanisms. Each portion is capable of separate and, to a large extent, independent variation. The primary isotonic urine is defined as the osmolar clearance, C_{osm}, calculated as $\frac{U_{osm}}{P_{osm}} \cdot V$.‡ The movements of solute-free water are measured as the free water clearance C_{H_2O}, calculated as $\frac{(1 - U_{osm}) \cdot V}{P_{osm}}$. V is the rate of urine flow and P_{osm} and U_{osm} are the milliosmolar concentration of plasma and urine. The free water clearance may have negative values, indicating a net concentrating process or positive values indicating a net dilution of the urine. At all times, the urine flow, V, equals the algebraic sum of C_{osm} and CH_2O. It is not within the scope of this analysis to discuss the factors which affect CH_2O, but important among them in the normal kidney are the level of neurohypophysial activity and the magnitude of C_{osm} itself. In the presence of maximal antidiuretic hormone activity and at low rates of C_{osm}, $(-)CH_2O$ is small and is limited by the osmotic ceiling of the urine (in man, 3–4 times the osmotic pressure of the plasma). As C_{osm} increases, $(-)CH_2O$ increases rapidly and appears to approach a limiting value, in man, of 5–7 cc/min at an osmolar clearance of 15–25 cc/min (see Brodsky, this volume). Further studies are essential to clarify this relation.

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† This term is primarily a mathematical concept and, as described in the text, denotes the volume and osmotic concentration which the bladder urine would have were no renal processes operative which specifically retain or excrete water. At this writing, we do not feel justified in considering the primary, isosmotic urine as truly descriptive of the fluid at any specific anatomic level of the nephron.

The urinary solute load determines C_{osm} and therefore the urine volume. The solute load during osmotic loading with a nonionized substance consists almost entirely of 2 fractions: (1) the loading substance itself, and (2) chlorides, mostly sodium and potassium. The rate of delivery of the loading agent into the urine is defined as rate of filtration of the agent less the rate of tubular reabsorption ($U \times V = P \times C_F - T$). T is specific and definable for each substance. For mannitol, T is small, 10 per cent or less of the filtered load, but with the exact limits not clear. For glucose, T is the maximal rate of tubular reabsorption of glucose (Tm_G), a term which is highly constant in each individual. For urea, T is a mathematical function of Pu and C_F/V, increasing as Pu increases, decreasing as C_F/V decreases. Since the rate of appearance in the urine of nonelectrolytes such as mannitol, glucose or urea is fairly easily stated in terms of glomerular filtration rate, plasma concentration and tubular reabsorption, the problem of osmolarity as a determinant of urine flow would be readily solved were it not for the fact that the leading solute is augmented by solute derived from impaired reabsorption of filtered sodium and chloride.§ No simple reason can be seen why this should be so. The tubular reabsorption of other constituents of normal plasma is impaired little or not at all. Electrical effects are not involved. There is no correlation with composition or molecular structure of the diuretic agent. The increased volume of urine flow consequent on increased solute load appears to be accommodated for in part by tubular dilatation and, to a much smaller extent, by an increase in mean tubular velocity of flow. There is no evidence that either dilatation or increased velocity can account for the increased NaCl excretion effected by loading with mannitol, etc. Indeed, most conditions which, like dilatation, increase intrarenal pressure (edema, venous congestion) are associated with increased rather than decreased sodium reabsorption.

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‡ Osmotic pressures of urine and plasma are determined by the methods described in (9) and (15).

§ Available evidence suggests that cellular potassium is facultatively exchanged for sodium, probably at some point in the distal tubule. Hence, a large proportion of the urinary potassium may represent sodium which might have been excreted were it not for this exchange process. Similar reasoning applies to excretion of ammonium and titrable acid, but these terms normally represent very little of the solute load during osmotic diuresis.

By elimination, and on experimental grounds, we may suspect that the effect of osmotic load on NaCl reabsorption is an osmotic one. It may be surmised that the diluting effect produced within the tubule by the retained osmotic agent together with its water impedes in some fashion the reabsorption of NaCl and that this diluting effect is less important in the case of other plasma constituents. Like the osmotic loading agent, the quantity of NaCl delivered into the urine depends on the balance between filtered load and tubular reabsorptive capacity; so that, in effect, osmotic loading depresses tubular reabsorptive capacity for Na and Cl. But the filtered NaCl load contributes equally to excretion, so that we are not justified in expecting that a given rate of excretion of an osmotic agent will necessarily "obligate" the excretion of a given quantity of NaCl, nor can we quantitatively express, at this time, the influence of the various factors which determine NaCl excretion during osmotic diuresis. Qualitative expression of some of them can be supplied:

1. At a given rate of excretion of a leading substance, e.g., urea, NaCl excretion will decrease if filtered NaCl load decreases and increase as filtered NaCl load increases (16).

2. The available evidence indicates that the 2 terms that determine filtered NaCl load ($C_F \cdot P_{Na}$) are qualitatively different (16).

3. Plasma NaCl concentration appears to be a potent load factor in determining NaCl excretion in that an increase in filtered NaCl load due solely to increase in plasma NaCl can result in a large increase in NaCl excretion (16).

4. Filtration rate appears to be a weaker load factor in determining NaCl excretion; i.e., an increase in load of the same magnitude as in paragraph (3) but due solely to increase in filtration rate causes a much smaller increase in NaCl excretion (16).

5. If intratubular dilution can be accepted as a major factor in

the inhibition of NaCl reabsorption and hence in promoting its excretion, it seems reasonable to express this effect in terms of a certain limiting plasma/tubular fluid concentration difference and to assign this effect to the proximal tubule.

6. Reasons have been given elsewhere (13) for considering as likely the existence of a second NaCl reabsorptive process lying distal to the proximal convoluted tubule. This process seems (*a*) to involve much smaller mass transfer than the proximal tubular process, but (*b*) to reabsorb NaCl over a larger concentration gradient. Therefore we may suspect that distal reabsorption is not materially affected by the presence of an osmotic loading agent.

7. An osmotic loading agent will deliver increased quantities of NaCl into the urine only insofar as, by depressing proximal reabsorption, it causes the NaCl load to exceed distal reabsorptive capacity. Circumstances can be defined in which even a large urinary load of an osmotic agent will have little or no effect on NaCl excretion.

8. Variability of the intrinsic or resting tubular NaCl reabsorptive capacity has not entered into the foregoing analysis. Such variations do occur in some circumstances. However, it is not clear that the variability occurs in the proximal or the distal or in both reabsorptive processes, and the occurrence of such variations during osmotic loading has not been established.

Osmotic diuresis produced by loading with ionized substances is much more complex than that produced by loading with nonionized substances. Sulfate is a good example. If sulfate is injected, all of the filtered sulfate, less that very small and fixed amount reabsorbed by the tubules, appears in the urine. In addition to sulfate, the double electrical charge on each ion forcibly extracts into the urine 2 cations; sodium provides the largest share of these. Thus, in contrast with nonionized osmotic agents, excretion of sodium plus potassium is always either equal to or greater than the equivalent rate of excretion of sulfate, and the minimal rate of total solute excretion under sulfate loading is easily predicted. When the sulfate load is large, however, chlorides appear in the urine, further enhancing the urinary solute load. The mechanism by which this is produced is not clear.

Selection of the appropriate osmotic diuretic for excretion studies should be made only after careful consideration of the advantages and disadvantages of each. Injection of hypertonic sodium sulfate produces an intense diuresis. It also produces withdrawal of cellular water, expansion of the extracellular fluid and decreases in serum concentrations of Cl^- and HCO_3^-. Isosmolar solutions of this salt, while avoiding cellular dehydration, produce a somewhat greater expansion of extracellular fluid volume. To these changes are added the difficulties of describing the mechanism by which sulfate impedes chloride reabsorption. Injection of carbohydrate diuretics produces the same general changes in the plasma as the saline diuretics except that sodium concentration is lowered in the same proportion as Cl^- and HCO_3^-. Injection of electrolyte to counter these effects results in cellular dehydration and overexpansion of extracellular fluid. The advantage of urea is that it does not as such change body water or electrolyte composition or distribution, so that these remain under the investigator's control. Disadvantages of urea are passive back-diffusion of some of the filtered load so that diuretic efficacy is diminished, and lack of a suitable experimental basis for comparison with other diuretics because of the extracellular location of most of these and the distortions of body water and electrolyte which they induce. For general studies of the specific effect on electrolyte excretion of heavy osmotic loads (more than 30 mOsm/l of plasma), substances other than urea cannot be recommended.

OSMOTIC DIURESIS: EXPERIMENTAL PROTOCOL

Our object in this experiment was study of renal function and factors determining electrolyte excretion under sustained osmotic (urea) loads with minimal changes in body fluid volume or composition.

a) *Solutions.*—A: Cr-PAH prime, as on page 181.

B: Cr-PAH sustaining solution, as on page 181.

C: Chloralose;[‖] and see *b*) below.

D: Urea prime. The urea may be made up as a 50 per cent solution and filtered. The desired quantity is added to a "synthetic urine" (solution E–1). This synthetic urine is divided into 2 portions. One contains the anesthetic dose of chloralose, 1 per cent, and some of the urea in a concentration not greater than 10 per cent. The other contains the rest of the urea prime in a concentration not greater than 20 per cent. If insufficient fluid is available for the priming dose of urea, a portion of the 2d synthetic replacement urine (E–2) should be used.

E: Synthetic urines. Three synthetic urines should be prepared in advance. The 1st (E–1) is estimated to match volume and composition of urine which will be excreted during the injection of the urea prime, allowing 20 min injection time for each 5 g of urea/kg. The 2d (E–2) matches the estimated volume and composition of urine which will be excreted during the 1st 10 min after

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[‖] Alpha-chloralose, pure; prepared by Kuhlman Frères, Paris, France; agent, H. P. Rossiger & Co., 55 Vandam St., New York.

injection of the prime. The 3d (E–3) approximates the mean composition of the urine which will be excreted during the experiment. This solution is added to infused urine to replace those portions which will be retained for analysis.

F: Cr-PAH replacement. 1.0 per cent Cr and 0.5 per cent PAH in distilled water.

G: 1.0M $NaHCO_3$—10 ml.

b) *Anesthesia.*—Anesthesia is desirable because of the transitory distress occasionally produced by the priming dose of urea and because of the long duration of many of our experiments. Chloralose is selected because of its minimal effects on blood pressure, heart rate, renal blood flow and filtration rate and the infrequency with which it modifies a water diuresis. Since chloralose is not very soluble in water, fluids which must be given as part of the experiment should be used as the solvent wherever possible. For the anesthetizing dose, the 1st portion of the urea prime may be used. Supplementary doses to sustain anesthesia may use some of the fluid replacements. Anesthetizing dose for dogs is 100 mg/kg in a 1 per cent solution. An additional 20 per cent of this dose should be available in a separate container in saline since an occasional animal requires more than the calculated dose.

Since chloralose rapidly hydrolyzes to glucose and chloral in hot water, it should be dissolved by rapid heating, with stirring, but it should not boil. As soon as dissolved, it is filtered rapidly through cotton and cooled under tap water to about 40 C. Prepare immediately before using.

The 1st half of the anesthetizing dose may be given rapidly, the rest more slowly. Ease of induction depends on calmness and relaxation of the animal. Chloralose depresses spinal reflexes poorly so that accurate estimation of the depth of anesthesia requires experience. As anesthesia deepens there is progressive loss of coordinated movement and of the more complex reflexes. When anesthesia is deep, there remain only isolated, erratic twitches and the myotatic reflexes. Animals may pass into respiratory paralysis at a time when muscle tone is good and the deep tendon reflexes are vigorous. We have not yet observed any significant effect of this anesthetic on renal function. To maintain a constant depth of anesthesia, the animal will need another anesthetizing dose in divided portions every 3–4 hr. This may be dissolved in some of the synthetic urine replacement (solution E–3).

c) *Procedure.*—The basic plan of this experiment is similar to that described for extracellular fluid expansion (pp. 181 ff). The animal is prepared and 3 control periods are secured. Because of the large urine flows produced and the more complex urine composition, the animal's own urine is reinfused to maintain water and electrolyte balance. During the control and equilibration periods, electrolyte and water losses are negligible and pump infusion of solution B maintains plasma concentrations of PAH and creatinine. At the end of the last control period administration is begun of the anesthetic and some of the urea prime in the 1st portion of solution E–1, followed by the rest of the urea in the 2d portion of E–1. These with solution E–2 replace salt and water losses during urea priming and the short (10 min) equilibration period that follows.

The urine is collected from termination of the last control to the end of the equilibration time in 2 time intervals of 15–20 min each. Rate of urine flow of the 1st portion is calculated. As the 1st period of clearance determination under osmotic load begins, infusion of the Cr-PAH sustaining solution is terminated and followed by infusion of the 1st urine sample at its calculated rate of flow. This in turn is followed by the 2d urine volume at its estimate rate of flow. This sequence of calculation of rate of urine flow and reinfusion of the urine at this calculated rate continues through the experiment.

It will be noted that a change from infusion of a Cr-PAH solution (solution B) to infusion of the animal's own urine does not interrupt the constancy in the minute infusion rate of Cr and PAH since their output has come to equal input during the equilibration and control periods. During replacement of the infusion by the urine excreted during the preceding 30 min., input of Cr and PAH continues to equal output, except for small, short term, self-correcting disparities. Animal, infusion flask and urine receptacle are converted into a closed system in which renal function may be measured for many hours with hardly perceptible variations in plasma Cr and PAH concentrations. Our data show no evidence of significant accumulation of non-Cr or non-PAH chromogens during 8 hr of urine reinfusion. The small amounts of Cr and PAH lost from the system are restored by solution F.

As each sample of urine is obtained, the following operations are performed as rapidly as possible: calculation of urine flow, sampling for analysis after mixing, filtration through washed cotton, replacement of estimated loss of urea and electrolyte in the analytical sample by addition of solution E–3 and loss of Cr and PAH by solution F[*] and addition of small amounts of solution G to maintain plasma CO_2 as indicated by analysis of the plasma at intervals during an experiment.

At the end of an experiment the animal remains under a heavy osmotic load so that it may become severely dehydrated when urine replacement is stopped. To protect against this, a urea-free modification of solution E–3 is infused in an amount of 40 cc/g of urea used in the priming dose.

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[*] ml sol. F added $= \dfrac{C\text{ (est)} \times P\text{ (est)} \times \text{ml in urine sample}}{10V\text{ (ml/min)}}$

Comment by William A. Brodsky

It is well known that changes in the volume and composition of extracellular fluids are occasioned by the injection of various loading solutes (1). However, the volume of urine during osmotic diuresis is dependent only on the number of osmotically active particles reaching the most distal portion of the nephron and is independent of (*a*) changes in volume of extracellular fluid, (*b*) changes in pH and CO_2 content of plasma and (*c*) alterations in composition of urinary solutes. The following comparative observations will lend support to the aforementioned hypothesis:

1. Urea, diffusing through all fluid compartments and producing little or no change in extracellular volume, evokes a response in urine flow and load almost identical to NaCl, a solute distributed through the extracellular space and producing a significant increase in extracellular volume (11).

2. Sodium bicarbonate, although provoking profound alkalosis, produces a diuretic response in dogs closely similar to that of NaH_2PO_4–Na_2HPO_4, a potent acidifying salt (10).

3. Sodium sulfate loading, with its attendant increase in plasma sodium level and suppression of chloride excretion, induces a response in urine flow and solute excretion similar to that of mannitol with its associated hyponatremia and marked increase of chloride excretion (11).

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IV. Determination of Water and Electrolyte Excretion during Osmotic Diuresis in Hydropenic Man

WILLIAM A. BRODSKY, *University of Louisville*

The responses of the kidney to solute loading during the hydropenic state provide an excellent means of measuring renal capacity for water and electrolyte conservation. During osmotic diuresis in man (12) and dog (15), a constant relation between urinary flow and excretion of solutes is observed with a wide variety of loading solutes. The pattern of electrolyte excretion is reproducible for any single loading substance. Rapoport and West (7) have shown that imposition of appropriate loads of the sodium salts of CNS, NO_3, HCO_3, PAH, SO_4, PO_4, $Fe(CN)_6$ and S_2O_3 in each case alters the pattern of chloride excretion in a specific manner, dependent on interionic antagonism and predictable from the Hofmeister series. On the other hand, loading with nonelectrolytes (mannitol, xylose, sorbose, glucose, sucrose) results in a pattern of electrolyte loss independent of interionic antagonism and representing a stress-load curve of electrolyte conservation during the hydropenic state. To standardize conditions for the assessment of water and electrolyte economy during osmotic diuresis, a nonelectrolyte such as mannitol is used as the loading solute. Mannitol has the advantages of innocuousness, availability and ease of determination.

PROCEDURE

a) Preparation of subject.—Water and food are withheld for 12–16 hr before the test so that urine flow is minimal and osmotic activity maximal immediately before injection of the loading solute. A priming injection of mannitol, in 25 per cent solution, is given intravenously in an amount calculated to add 20–40 mOsm/l of distribution volume, followed by a maintenance infusion in the amount of 1 per cent of the priming dose/min. If simultaneous measurement of renal plasma flow is desired, PAH is added to the infusion fluids in quantities sufficient to maintain a plasma level of 3 mg./100 cc. (The amount of PAH used to measure effective renal plasma flow is too small [concentration of 3 mg/100 cc is but a fraction of 1 mOsm/l] to interfere with electrolyte excretion occasioned by the large osmotic loads [20–40 mOsm/l] of mannitol, etc.)

Sample Protocol for Mannitol Loading

Patient: John Doe, age 25 years, weight 70 kg
Level of mannitol desired: 20 mOsm/l

Assumed vol of distribution: 0.2×70 kg body weight
Amt of mannitol required: $20 \times 14 = 280$ mOsm or 51 g; or 204 cc of 25% solution
Disappearance rate: 1% of amt in the body/min
Corrected priming dose: 214 cc
Maintenance dose: 2.04 cc/min
Correction for amt of mannitol excreted during a 10 min period allowed for priming injection: $204\ (0.5) \times (0.01)\ (10) = 10.2$ cc

b) Constant infusion machine.—For this procedure, the use of a constant infusion pump is most desirable. The apparatus used in our laboratory employs 2 electrically regulated pistons which push the plungers of 2 calibrated syringes, the smaller of which is used for slow infusion of potent pharmacologic agents.*

c) Collection of urine and blood samples.—Urine is collected by catheterization in females and by voluntary voiding in males. Before loading, urine is collected over 20–30 min intervals for 2 periods. After the 10 min priming injection, 15 min is allowed for equilibration of body fluids toward a steady state. The equilibration urine, designated $X - 1$, represents the time interval from the last preliminary period to the end of equilibration. After equilibration, with urine flows at diuretic levels, the collection periods are of 10–20 min duration. Three or 4 such samples are collected. Blood samples are drawn at approximate midpoints of collection periods.

The entire procedure is readily tolerated by the patients and lasts no longer than 2 hr.

d) Definition of terms.—1. Osmotic Activity = concentration of the total number of osmotically active constituents in urine calculated by the degree of depression of freezing point or vapor tension (Raoult's law).

2. Effective Osmotic Load = the total number of osmotically active particles excreted each minute; the product of the minute volume and osmotic activity of urine.

3. Flow-Load Relationship = the relation between minute volume of urine and effective osmotic load. For example, in the hydropenic state, a constant volume of urinary water is required for excretion of a given osmotic load.

4. Particulate Concentration = the total number of particles per unit volume of solution as determined by the sum of observed concentration of each solute. Particulate concentration will differ from osmotic activity (especially in electrolyte-containing solu-

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* The most recent modification of this device is obtainable from Engineering Specialties Company, 7113 Miami Ave., Madeira, Ohio. Another is the Bowman infusion pump, obtainable from Process and Instruments, 60 Greenpoint Ave., Brooklyn 22, N.Y.

tions) to an extent predictable by the degree of dissociation of the salts and forces of interionic attraction. The term is synonymous with "true total osmolarity," or the osmolarity calculated at infinite dilutions.

e) Determination of osmotic activity.—According to Raoult's law, the freezing point depression of a dilute aqueous system is proportional to the mole fraction of solute in solution and therefore to the osmotic activity of the solution. The principle and technique of measuring freezing point depression of solution have undergone little change since first used by Beckman in 1888.

URINE.—The apparatus for *determination of freezing point depression,* commonly used, is the Cryoscope.* It consists of a large test tube of 100 ml capacity placed in a steel jacket surrounded by a Dewar flask. An aliquot of urine is placed into the test tube fitted with a stirrer and a Beckman thermometer graduated in hundredths of a degree (range, +2 to −2° C). The Dewar flask is $^1/_3$ to $^1/_2$ filled with ether. Water suction applied to the ether-filled jacket vaporizes the ether, thus cooling the solution in the inner tube. By gradual lowering of the temperature of the liquid and stirring to avoid excessive supercooling, the freezing point, at which the temperature remains constant, can be observed. Osmotic activity, expressed as mOsm/l, is obtained by applying the formula

$$\text{Osmotic activity} = \frac{\text{freezing point depression} \times 1{,}000}{1.86}$$

where 1.86 is the molal depression constant for aqueous solutions.

PLASMA.—If sufficient plasma is available, direct determination of the freezing point is possible. This technique, however, has not in my experience been highly accurate in protein-containing solutions. Consequently, osmotic activity of plasma is estimated from the sum of the determined solutes from the equation

$$\text{Osmotic activity} = 2(\text{Na} + \text{K}) - 8 + (\text{urea}) + (\text{loading solute}) \times \gamma$$

where $\gamma = 0.925$, the assumed activity coefficient of plasma, and the terms in parentheses are in the following units: Na and K, in mEq/l, so that $2(\text{Na} + \text{K}) - 8 =$ the osmolar concentration of electrolytes; urea, loading solute and osmotic activity, in mOsm/l.

f) Measurement of osmotic limitations of the kidney.—Figure 1, a sample plot of flow vs. load, illustrates the dependence of urinary flow on the effective osmotic load during osmotic diuresis with mannitol in man. Since similar patterns of flow load have been observed with many different loading solutes, it has been postulated that water reabsorption must be the final event of tubular function during hydropenic diuresis (1, 12, 15). Thus the flow-load curve may be used to delimit distal tubular function. Data falling to the left of the curve indicate a defective capacity for water conservation in that more water than normal is required for excretion of a given load.

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* Manufactured by Eimer and Amend, New York, N. Y.

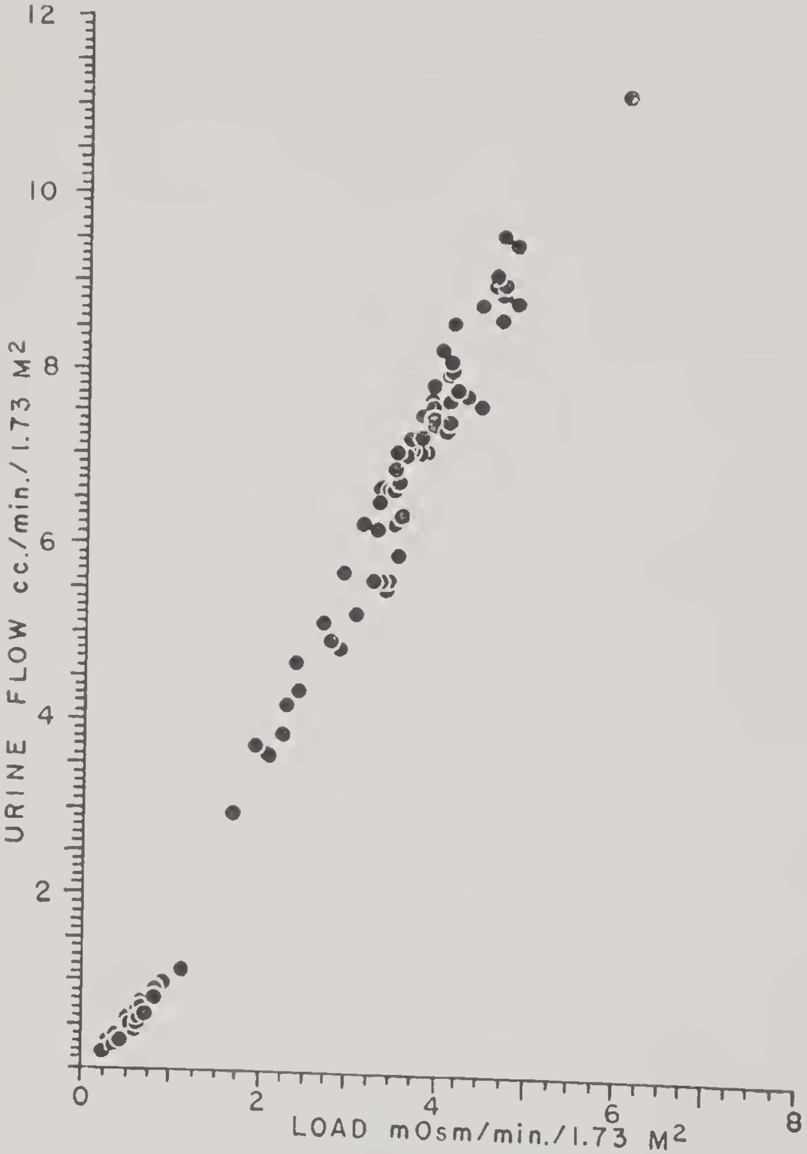

FIG. 1.—Urine flow vs. effective osmotic load in 16 normal subjects before and during mannitol diuresis. (Data from Brodsky, Rapoport, Graubarth and Levkoff, unpublished data.)

In the production of hypertonic urine, the osmolarity of which exceeds that of body fluids, a water economy (H_2O_{Ec}) must result. It may be expressed as (2, 7)

$$\frac{\text{Effective osmotic load}}{\text{Osmotic activity of plasma}} - \text{urine vol} = H_2O_{Ec}$$

The value of the 1st member signifies the volume of isotonic body fluid cleared by the kidney. It may be interpreted also as the volume of isotonic fluid presented to the distal tubule after proximal iso-osmotic reabsorption. The water economized distally during osmotic diuresis is distributed among the body fluid compartments so that they tend to return toward the preloading level of osmotic activity. Figure 2 illustrates the relation of water economy to urine flow during mannitol diuresis in man. A level of 5–7 cc/min is reached at flows exceeding 4 cc/min, with small change at higher flows. The intensity of water economy, i.e., the amount conserved/cc. of urine elaborated is greater, the higher the osmolarity of the urine. Thus, at extremely high flow, when the osmolarity of urine approaches that of water, intensity of water economy will approach very low levels.

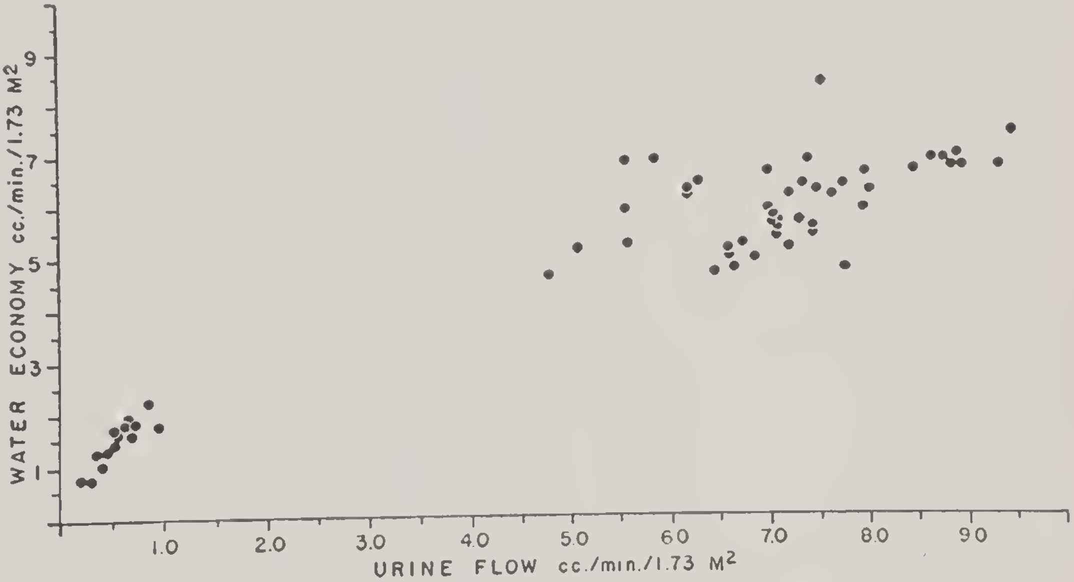

Fig. 2.—Water economy vs. urine flow.

g) Measurement of electrolyte excretion.—1. ASSESSMENT OF COMPOSITION OF URINARY ELECTROLYTES.—The rate of sodium and chloride loss during osmotic diuresis in man is almost linearly related to urinary flow or load (2, 9). Since total particulate and effective osmotic loads are almost identical when the predominant urinary solute is a nonelectrolyte, the plot of electrolyte losses vs effective load will depict graphically the composition of urinary solutes over a wide range of excreted loads. For example, Figure 3 illustrates the constant patterns of sodium, chloride and potassium loss/unit of excreted solute in 16 normal subjects and in 3 patients with diabetes insipidus under similar loading conditions. The similarity of the pattern in diabetes insipidus to the normal indicates that solute excretion proceeds in a perfectly normal manner despite the gross defect in water excretion (1).

From data on solute composition and flow-load relationship in the urine, one may localize approximately the site of renal defects in various clinical states. For example, in diabetes insipidus, the finding of an abnormal flow-load relation with a normal composition of urinary solutes suggests a distal portion of the tubule as the main site of renal dysfunction. In patients with tuberculous meningitis (10) or in splanchnicotomized dogs (3) the finding of normal flow-load relations with distorted composition of urinary solutes suggests proximal tubular defects. It is of interest that neither the pattern of electrolyte loss nor flow-load relations have been correlated with the level of glomerular filtration (1, 7).

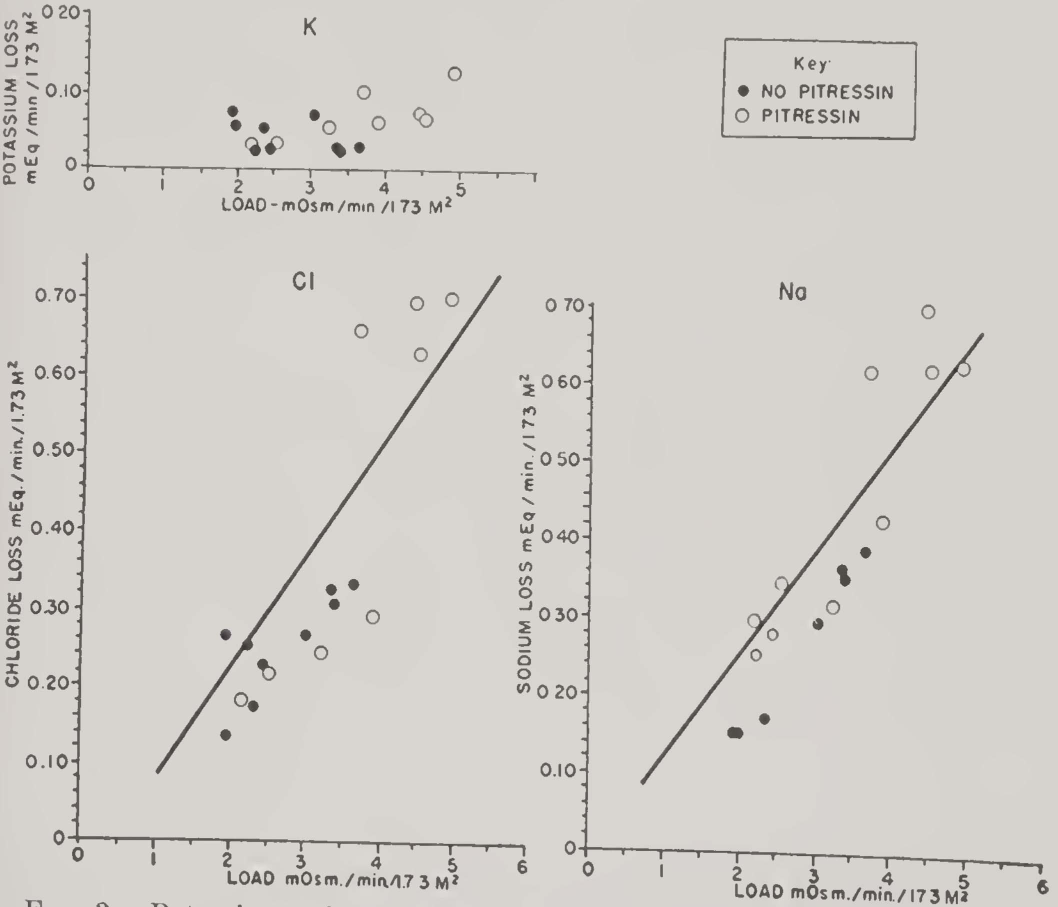

FIG. 3.—Potassium, chloride and sodium losses vs. osmotic load during mannitol loading. Open and solid circles refer to 3 patients with diabetes insipidus with and without Pitressin therapy, respectively. The lines represent calculated regressions for sodium and chloride loss against osmotic load in 16 normal human subjects. (See ref. (1).)

2. CONCENTRATION GRADIENTS BETWEEN PLASMA AND URINE.—Another approach to the study of electrolyte excretion consists in

observations on concentration gradients from plasma to urine reached by given constituents. For example, during infusions of hypertonic saline the concentration of urinary chloride reaches levels as high as 300 mEq/l, whereas during water diuresis the concentration falls to 5–10 mEq/l. In certain clinical states of "salt-losing," loading with hypertonic saline fails to increase the concentration of urinary chloride above 150 mEq/l, whereas water loading fails to depress urinary chlorides to low levels (10).

3. WATER DIURESIS.—Water diuresis is a condition during which electrolyte conservation proceeds at maximal levels (4, 5, 6, 14). Studies of water and solute excretion under such conditions should yield data describing capacities for both electrolyte conservation and dilution functions of the kidney.

4. BALANCE STUDIES.—These may be used as a measure of renal capacity for electrolyte conservation in the presence of hyper- or hypoelectrolytemia, with controlled intakes of salt (10, 11, 13).

5. THERMODYNAMIC WORK.—Calculations of the "ideal" or thermodynamic energy output of the kidney, under conditions of large osmotic loads, may serve as a useful parameter of the renal capacity for solute excretion. Such calculations have limited value because osmotic energy is but a small fraction of the total energy output of the kidney; nevertheless the pattern of osmotic work during solute loading is constant, approaching a biologically maximal value (2, 8). Calculations of work necessitate addition of the minimal free energy necessary to effectuate the observed concentration gradients of all solute constituents from plasma to urine. Applying the formula of von Rhorer (13)

$$W = VRT\,(\mathrm{Uln}\ U/P\ P - U)$$

where R is the gas constant; T the absolute temperature; W, the energy expenditure in g-cal/min; V the corrected minute volume of urine, and U and P the molar concentrations of each solute in urine and plasma, respectively. It has been found that osmotic work in hydropenic man is of the order of approximately 0.4 g-cal/min/-1.73m^2 (8) and that it rises to a level of 3–4 g-cal after loading (9). Further studies are needed to assess the value of such calculations in various disease states.

NOTE.—This section was reviewed by Robert F. Pitts.

Comment by Clark D. West

There is little to add to the description of the procedure given here. It should be emphasized that observations on normal subjects have shown that for reproducibility of the flow-load relation a truly hydropenic state should exist. The most reliable indicator of presence of hydropenia is a low rate of urine flow in the preliminary periods (0.5 cc/min or less) and a high osmolarity (greater than 1,000 mOsm/l).

Observations on urinary composition and volume after solute loading, first made by McCance and later and more extensively by Rapoport, have led to new and interesting concepts of the process of urine formation. As Dr. Brodsky indicates, these studies have made clear a functional division of the tubule as concerns water and solute reabsorption. Later work, applying the technique to patients with diabetes insipidus, has raised important questions as to the renal mechanism for the production of a dilute urine.

The possibilities for further use of this method of study are numerous. The flow-load relation during osmotic diuresis would seem well adapted to the study of the action of diuretics. Comparatively little work has been done to investigate the flow-load pattern in the numerous clinical conditions in which renal function is abnormal. Perhaps even more valuable will be the application of solute loading to study of the multiple factors which influence electrolyte excretion. The stable and reproducible electrolyte composition of the urine during osmotic diuresis, difficult to achieve by other methods, has already been fruitfully employed in study of the effect of splanchnicotomy on renal electrolyte control and to some extent in study of the electrolyte retention accompanying edema. The procedure is innocuous especially when nonelectrolyte loading solutes are used, and, if elevation of plasma osmolarity is not greater than 20–30 mOsm/l, produces no more discomfort than the more commonly used tests of renal function. Its clinical applications have been too meager to permit comment on its value as more than a research tool.

Comment by Laurence G. Wesson, Jr.

The procedure might perhaps be simplified for clinical purposes if it could be shown that subcutaneous injection of Pitressin (say, 500 mU) could substitute for the natural hydropenic state.

The postulate that "water reabsorption is the final event of tubular function during hydropenic diuresis" implies that water reabsorption is performed distally to acidification and ammoniation. Can this be supported?

In the discussion of water economy, the terms "volume of isotonic body fluid cleared by the kidney" and "water economy" correspond respectively to our terms "osmolar clearance" and "negative free water clearance."

The term "pattern of electrolyte loss" is not self-explanatory. If by it is meant the ratio of rates of electrolyte excretion to excretion of loading substances, we have found definite alterations in this term in the dog, in which glomerular filtration rate is more readily varied than in man.

Last, can measurements of urinary specific gravity substitute for cryoscopy in clinical assessment of flow-load function?

Comment by Dr. Brodsky

We have no data on the use of Pitressin as a substitute for hydropenia. That water reabsorption occurs distally to the functions noted is indicated by the constancy of flow-load relationships under loading with both alkalinizing and acidifying salts.

The "pattern of electrolyte loss" is the rate of excretion of a specific electrolyte in relation to total solute excretion.

I doubt that measurements of specific gravity can be substituted for those of freezing point depression, since the term desired is the relationship between urine volume and moles rather than grams per unit volume.

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V. Bioassay of Antidiuretic and Diuretic Substances

ASSAY OF ANTIDIURETIC SUBSTANCES IN URINE

J. MAXWELL LITTLE, *Bowman Gray School of Medicine*

During development of a bioassay procedure for either an antidiuretic or a diuretic substance the principal difficulty encountered has been the insuring of a relatively constant state of hydration in the test animals, as well as a reproducible response to a fluid load presented at or about the time of administration of the material being assayed. Several methods of hydration and analysis of the response have been published for the white mouse, white rat and dog.

The rat technique for bioassay of the activity of preparations of the posterior pituitary was introduced in a quantitative procedure by Burn (1). Since then, many modifications have appeared. Ham (5) studied several of these modifications (3, 8, 12, 13) and found that the method of hydration used by Gilman and Goodman (3) and the method used by Krieger and Kilvington (8) for expressing the results of the assay gave the most reproducible figures in control experiments. Ham and Landis (6) have incorporated these principles in the bioassay procedure given here.

ASSAY USING RATS

A stock of male albino rats is kept in small groups in a room maintained at an even temperature. The animals are fed a weighed amount of food in excess of the expected daily requirements. The daily consumption is determined by weighing the amount not consumed. Animals not consuming a normal amount of food are not included in the assay groups. Fresh water is kept constantly available.

Before each assay 18 rats are fasted for 12 hr but are allowed to have water ad lib. The next morning the rats are placed, by groups of 3, in a bank of 6 metabolism cages. The cages are set over 9 in. chemically clean glass funnels which drain into 50 cc graduate cylinders. Small conical screens in the apexes of the funnels prevent feces from falling into the graduates. The rats are weighed and distributed, if possible, so that the total weight of the 3 rats in each group does not differ by more than 10 per cent. Two of the 6 groups are used as controls, leaving 4 groups for duplicate assays of 2 unknown solutions, or 1 unknown and 1 standard preparation of an antidiuretic substance.

Each rat is given by gavage a priming dose of 0.2 per cent NaCl solution equivalent to 2.5 per cent of the body weight. Two hr after the priming dose was given, all urine excreted up to that time is discarded. Each rat is then given a hydrating dose of 0.2 per cent NaCl solution equivalent to 5 per cent of the original body weight. Simultaneously the solution to be assayed is injected intraperitoneally in a volume totaling 1 cc/100 g of rat. The controls are given similar gavage and they receive intraperitoneally either 0.9 per cent NaCl solution or distilled water to match the composition of the solutions being tested.

Starting then at zero time for each cage, the cumulative volume of urine excreted by each group is recorded at 15 min intervals for 3 hr. The recorded volumes are computed in terms of the amount excreted per 100 g of rat and plotted against time for each cage on a chart in which 1 in. represents 30 min and 1.0 cc of urine. To express the results by a single number, the areas of these curves are measured in square inches, using a planimeter.

Critique.—In 130 experiments the variation between 2 control cages, observed on the same day, averaged 1.56 sq in., with a maximal variation of 5 sq in., which occurred in only 2 experiments (6). The method is accurate for doses of 0.5–10 mU of Pituitrin/100 g of rat. As long as the rats eat well and maintain their weight they may be used for not more than 3 assays at intervals of 10 days or more.

It has been suggested (5, 13) that Pituitrin solutions may be assayed more accurately by measuring the effect of the preparation on the total chloride excretion, expressed as microequivalents/100 g of rat/3 hr, than by measuring the effect on water excretion. With the chloride excretion procedure it was shown (5) that the accurate range for Pituitrin solutions was from 0.1 to 100 mU/100 g of rat. However, since there is some doubt whether or not the antidiuretic and chloruretic effects of pituitary extracts may be attributed to the same molecular substance (4, 10, 11) and since it has been reported (6) that an antidiuretic substance found in human urine was without chloruretic effect, it would appear reasonable to restrict the assay procedure to the renal function being studied, e.g., water reabsorption or chloruresis, as the case may be.

ASSAY USING DOGS

The principle involved in the procedure of Hare *et al.* (7) is the determination of changes in the creatinine urinary-plasma (U/P) ratio resulting from the action of an antidiuretic substance in dogs with diabetes insipidus.

Permanent polyuria is produced in female dogs by surgical transection of the pituitary stalk. The dogs are trained to lie quietly on padded boards with loose restraining thongs. Food is removed 24 hr and water 3 hr before the experiment. Three g of creatinine in 100 cc of water is administered by stomach tube 2 hr before the assay. Urine is collected by a retention catheter (no. 16 Pezzer), which is left in the bladder throughout the experiment, draining into a volumetric flask. The bladder is emptied at the end of each clearance period by washing with air. Blood samples are collected at the midpoint of the 1st clearance period and hourly thereafter. Creatinine is determined on tungstic acid filtrates of plasma and diluted aliquots of urine by the alkaline picrate method (2), and the concentration of creatinine in the urine is compared with that of plasma at the midpoint of the clearance period, obtained by interpolation on a plasma concentration-time curve constructed from the data on the hourly blood samples.

A control period of 20 min is run, the material being assayed is given intravenously and two 20 min assay periods are run. The assay of an unknown should be preceded and followed by the same dose of a standard antidiuretic preparation (Pituitrin or Pitressin). The usual assay requires 3 hr and 20 min.

The results are calculated as percentile change in the creatinine U/P ratio, taking as the basal creatinine U/P ratio the average of the ratios for the control periods immediately preceding and following the assay of the unknown or the standard. Presence or absence of antidiuretic material may be detected by use of a single assay; however, assays are usually run in duplicate or triplicate. When duplicate assays agree within 0.3 mU, the average is considered acceptable; when 2 of the triplicate assays agree within 0.3 mU and the 3d is erratic, the average of the 2 is considered acceptable.

The useful range of dosage for a standard is 0.3–3.0 mU. Doses of 0.1 mU can be detected, but they may be estimated somewhere between 0.05 and 0.2 mU, and doses of 3.0 mU or more never assay greater than 3.0 mU. If an unknown gives a value larger than 2.0 mU the assay should be repeated with a smaller dose.

Only freshly collected urine specimens should be used and even when they are clear the dogs may retch. These reactions do not affect the assay. Urine from patients or animals with urinary tract infections may cause prolonged oliguria or anuria. An occasional urine may show a decided diuretic effect (see also (9)) which may prohibit the evaluation of subsequent injections. When the urine flow of the assay dog is less than 1.5 cc/min, the maximal dose of the standard is 2.0 mU, and the dose decreases as the urine flow falls; therefore it is essential that the basal urine flow be at least 1.5 cc/min, and one of 2–5 cc/min is preferable.

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CONCENTRATION OF ANTIDIURETIC IN URINE

LOUIS B. TURNER *and* ARTHUR GROLLMAN, *Southwestern University*

The role of the posterior pituitary hormone in regulation of water excretion by the kidney is well established. Because of this relationship, investigators of renal function are often interested in determining the antidiuretic action of the urine in order to evaluate the role of the posterior pituitary in modifying water exchange. It must be emphasized that the substance in the blood and urine which exerts antidiuretic action has not been demonstrated to be identical with the hormone derived from the neural lobe of the hypophysis. In fact, the urinary principle differs in many respects from the hormone, lacking, for example, the chloruretic action of the latter. This has been interpreted by Grollman and Woods (4) as being due to oxidative processes in the body which abolish the chloruretic without affecting the antidiuretic action of the hormone. However, as demonstrated by Croxatto, Rojas and Barnafi (2), digestion of globulin with pepsin results in the production of a potent antidiuretic compound. It is quite possible, therefore, that much of the antidiuretic substance encountered in the urine or other body fluids is of extrapituitary origin.

The available methods consist essentially in purification of the urine to a degree which permits its injection into hydrated rats and determining the antidiuretic action thus induced. Earlier procedures involved the concentrating of the urine and dialysis of the concentrate. The latter process, however, entails a loss of a variable degree of antidiuretic activity due to dialyzability of the active principle (3). To avoid this source of error and at the same time to effect a sufficient purification and concentration of the active factor, Grollman and Woods (4) have utilized the following procedure.

PROCEDURE

The sample of freshly collected urine is filtered and brought to pH 4.5–5.0 by the addition of acetic acid. One g of absorbent powdered charcoal (Darco activated carbon, grade G-60, or other grades of charcoal suitable for adsorption from aqueous solutions) is added for each 100 cc of urine and the mixture agitated at intervals for several hours. It is placed in the ice-chest overnight and the supernatant urine discarded. The charcoal is collected on a Buchner funnel, washed with small amounts of distilled water to remove adherent urine, drained and transferred to a cylindrical centrifuge tube. The charcoal on which the antidiuretic hormone is quantitatively adsorbed is suspended in glacial acetic acid (5 cc/g of charcoal), agitated well and precipitated by the addition of 10 vol of a mixture of equal parts of absolute ethyl alcohol and petroleum ether. The amount of precipitate formed at this step of the procedure is so minute that it is scarcely perceptible, so no attempt should be made to remove it by filtration. However, after standing in the ice-chest overnight it deposits on the walls of the centrifuge tube, and any undeposited material is removed by centrifugation, the supernatant fluid being discarded. After drying in a vacuum desiccator at room temperature for at least 1 hr to remove the organic solvents, the precipitate is dissolved in a small amount of distilled water and filtered.

It is then assayed by the bioassay procedure of Burn (1) as modified by Ralli *et al.* (5). Four adult male rats, weighing 120–240 g, fasted 12–18 hr but allowed water ad lib up to the time of the assay, are placed in a metabolism cage. To each rat is administered 5 per cent of its body weight of lukewarm tap water by stomach tube. Immediately, thereafter, 1 cc of the solution to be assayed is injected intraperitoneally. The volume of urine excreted is recorded at 15 min intervals, and the time required for excreting 50 per cent of the administered water is used as an index of the antidiuretic potency of the injected extract.

NOTE.—This section was reviewed by J. Maxwell Little.

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ASSAY OF DIURETIC SUBSTANCES IN URINE

J. MAXWELL LITTLE, *Bowman Gray School of Medicine*

ASSAY USING RATS (*1*)

Male albino rats weighing 140–240 g are fasted and deprived of water for 18 hr before the assay. The animals are divided into subgroups of 4 each and placed in small cylindrical metabolimeter cages, 10 in. in diameter and 11 in. high, which rest on suitable glass funnels. The urine is collected by drainage into graduate cylinders. A small wire screen inserted in the neck of the funnel prevents feces from passing into the cylinder. Two subgroups (8 rats) are used for each dose of a standard or unknown substance.

Preliminary experiments with an unknown substance are done on 1 day, using 5 groups of 8 rats each of approximately the same weight. The control group is given 25 cc/kg of 0.9 per cent NaCl by stomach tube, containing no drug, and the other 4 groups are given geometrically increasing doses of the substance being tested. Thus the right dose range is determined in very few experiments. If the drug is to be administered orally it is dissolved or suspended in 0.9 per cent NaCl and given in a dose of 25 cc/kg. If the drug is to be administered parenterally the same saline dose is given and the drug is injected immediately afterward. The rats remain in the metabolimeter cages without food or water for the duration of the assay. With many substances the assay may be terminated at the end of 5 hr, since the rate of urine flow will show a sharp decline by this time; but with some substances, such as mercurial diuretics, it is suggested that the assay time be extended to 24 hr. On termination of the assay, residual bladder urine is expelled by pulling at the base of the rat's tail.

For the estimation of potency, 5 groups of 8 rats are used on 1 day. One group receives saline alone, 2 groups receive 2 suitable doses of urea (e.g., 0.75 and 1.5 g/kg), which is used as a standard and is dissolved in the saline, and the remaining 2 groups receive 2 suitable doses of the unknown substance chosen so that their actions will fall within the same range as those of urea. Three or 4 such experiments will produce enough data for a sufficiently accurate estimate of the mean activity (potency) of the substance relative to urea.

The volume of urine excreted during the assay time is expressed as per cent of the liquid administered, and it is called the diuretic effect. The best-fitting straight line for each set of data is calculated from the regression equation

$$y = \bar{y} + b(x - \bar{x})$$

where $\bar{y}$ is the mean value of y (log effect), $\bar{x}$ the mean value of x (log dose) and b the regression coefficient determined from the equation

$$b = \frac{\Sigma y(x - \bar{x})}{\Sigma(x - \bar{x})^2}$$

When the dose-effect lines are parallel, within the experiment error, the distance along the abscissa between the line for urea and that for the unknown substance gives the logarithm of the diuretic activity of the substance relative to urea, with an assigned potency of 1.0. When the dose-effect lines differ significantly in slope the relative potency is computed at a specified level of log per cent excretion. With this assay procedure some diuretic potencies, relative to urea 1.0, were: Salyrgan, 400; theophylline, 115; caffeine, 32, and ammonium chloride, 2.7 (1).

Modification.—In our laboratory (2) a modification of this procedure has decreased considerably the variability of the control per cent excretion rates. Food is removed 18 hr before the assay, and at that time the rats are given a hydrating dose of 25 cc/kg of 0.9 per cent NaCl. Water is left available until 1.5 hr before the beginning of the assay. From this point the procedure we have used is identical with that of Lipschitz *et al.* (1). Comparisons of data obtained by us using this modification with those obtained using the Lipschitz procedure showed the mean coefficients of variation for 6 groups studied on 6 separate days at several time intervals to be, respectively: 5 hr, 16.4 and 21.9 per cent; 10 hr, 11.0 and 18.1 per cent; 24 hr, 12.0 and 14.4 per cent. We have also found it advantageous to keep our animals in a room with temperature control during the assay period.

ASSAY USING DOGS (*2, 3*)

Female dogs weighing 6–12 kg are prepared for easy catheterization by an episiotomy, and they are trained to lie quietly on the animal board with loose restraining thongs. At 4:00 P.M. on the day before the assay is to be carried out they are given 25 cc/kg of tap water by stomach tube as a hydrating dose, and food is removed. Water is left available until 8:30 A.M. on the day of assay. At 10:00 A.M. the animals are catheterized, the bladder is emptied and the urine is discarded, the catheter being left in place. At the same time a tap water load of 25 cc/kg is given by stomach tube. At 11:00 A.M. the 1st collection period is ended and the unknown substance administered. At noon the 2d collection period is ended and the animals are returned to standard metabolimeter cages without food or water. At 4:00 P.M. the bladder is emptied by catheterization, ending collection period 3. The total urinary excretion for the 3 periods is used to calculate the excretion in terms of cc/kg for 6 hr. The duration of assay may be prolonged to accommodate a long-acting diuretic substance. The animals are kept in a room maintained at approximately 22 C during the assay.

The control rate of excretion, giving 2 ml of distilled water intravenously instead of a diuretic substance, is determined in 8 or more experiments for each animal. We have found it convenient to use a group of 12 dogs, 6 of which are used on alternate days for assay. Sixteen control experiments each on 10 dogs gave the following mean urine flows in cc/kg, with the corresponding standard deviations, for the 6 hr period: 25.6 ± 0.58, 25.8 ± 0.79, 25.6 ± 0.50, 25.5 ± 0.32, 25.6 ± 0.28, 26.9 ± 1.04, 25.7 ± 0.74, 26.1 ± 2.42, 25.4 ± 1.31, and 25.3 ± 0.68. These control excretion rates have not varied by more than 1 SD from the original mean, with frequent determinations, over a period of several months.

The assay result is expressed as the ratio of the total excretion in cc/kg in excess of the mean control excretion for the same period of time to the sum of twice the standard deviations of the mean controls:

$$\text{Diuretic ratio} = \frac{\Sigma(\text{exper. excretion} - \text{mean control excretion})}{\Sigma(2 \times \text{SD of mean control excretions})}$$

This expression permits one to determine whether or not the increase of urine flow caused by a substance is significant, and it gives some indication as to the magnitude of the significance.

Modification.—To compare an unknown substance with a standard substance (urea or Salyrgan), the procedure may be modified by administering the unknown parenterally at the time of administration of the water load, or dissolved in the water load, to 3 animals, and the standard administered in the same manner to 3 more animals on the same day. Two or 3 such experiments will suffice for an assay. The assay result is expressed as the *diuretic response,* which is the average excretion in cc/kg in excess of the control mean excretion for each dog

$$\text{Diuretic response} = \frac{\Sigma(\text{exper. excretion} - \text{mean control excretion})}{\text{number of dogs}}$$

and the *diuretic potency* is expressed as the ratio of the diuretic responses for the unknown and for the standard.

NOTE.—This section was reviewed by Robert Gaunt.

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DETECTION OF ANTIDIURETIC SUBSTANCES IN BLOOD

ROBERT GAUNT, *Syracuse University**

Many attempts have been made to detect antidiuretic activity in blood, largely with the purpose of identifying circulating posterior pituitary antidiuretic hormone (ADH). Some successes have been reported (2, 14, 17–19, 22, 23). Failures to obtain any activity from blood have also been frequent (6, 10, 13, 15). There is practically no agreement, however, among those who have found activity in blood as to whether the active agent is or is not ADH. The problem is inherently complex because of the low concentration of active material in blood, the lability of ADH in blood, and the possible presence of more than 1 antidiuretic agent and of interfering antagonist diuretic substances. If fractionation procedures are used, there is also the question of whether to separate materials of low or high molecular weight (1).

Recently more promising results have been obtained by a method of ultimate simplicity, namely, the use as a test material of fresh whole blood, serum or plasma. This technique was probably first used successfully by Griffith and co-workers (9, 20). Although uniform results and interpretations have not resulted from such methods, antidiuretic activity in blood can be detected and in certain instances has been due almost certainly to the presence of ADH.

The method described here resulted from the efforts of my colleagues, Birnie, Eversole and Jenkins (4, 5), to find a humoral antidiuretic substance (ADS) which might account for the failure of adrenalectomized rats to show normal water diuresis. In this work they found that normal rat serum was antidiuretic, and subsequent tests suggest, but perhaps do not prove, that this activity is due to ADH.

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* Present address, Ciba Pharmaceutical Products, Inc., Summit, N. J.

PROCEDURE (*5*)

Rats are anesthetized with ether, the thorax is opened and blood drawn directly from the right heart. Similar results are obtained with blood drawn under sodium pentobarbital anesthesia or by direct cardiac puncture without anesthesia. The blood is immediately centrifuged and an attempt made to get the serum injected into test animals within 5 min from the time it is drawn. If plasma is desired the blood is collected in syringes containing minimal effective amounts of heparin. Some activity is lost within 30 min and most or all of it is lost if decanted serum samples remain in a refrigerator overnight, but systematic studies of the rate of inactivation of the ADS have not been made.

Groups of 6 male rats, approximating 200 g body weight, are deprived of food but allowed access to drinking water for 18 hr before hydration. They are kept in small individual metabolism cages throughout the experiments. Three hydrating doses of water, warmed to body temperature, are given at hourly intervals by stomach tube.[†] The water dose is 3 ml/100 sq cm of body surface.[‡] Slessor (21) prefers to use 0.2 per cent NaCl as a hydrating fluid rather than water.

One hour after administration of the 2d dose of water the urine volume is measured. Any rat is discarded which during the 2 hr hydration period has a urine volume 50 per cent more or less than the mean of the group. The rats with normal diuresis are then given intraperitoneal injections of 1 ml samples[§] of the test material and a 3d dose of water is administered. Thereafter the urine output is measured at 30 min intervals for 3 hr. The water excreted during this period is calculated as the percentage of the total water given (in 3 doses) minus that excreted before injection of the test material. The percentage of water excreted at 90 min after the injection of the test material was adopted as the definitive figure, and in ordinary experiments there is little to be gained by continuing observations beyond that time.

The preliminary hydration period, which in principle has been used previously by Heller and Urban (12), permits the exclusion of animals likely to give aberrant responses and, in our opinion and that of Slessor (21) (although complete experimental proof is not available), adds considerably to the sensitivity of the test animals.

[†] The stomach tube is made of an 18 gauge, 2$^1/_2$ in. hypodermic needle with a polished metal knob of 2.3 mm diameter at its tip. With practice, these tubes can be used with great facility.

[‡] The surface area is calculated as per Benedict (3) by the formula: $S = 9.1 \times W^{2/3}$, in which S is the body surface and W the body weight. This method of calculating water loads equalizes the diuretic response to water in rats of conspicuously different sizes. In experiments such as these, in which rats of standard sizes are used, a dose of 4 or 5 ml/100 g body weight would certainly work as well.

[§] Heller and Ginsburg (11) use 1 ml/100 g body weight, and for obvious reasons that is probably a preferable procedure.

Precautions.—The precautions we have observed most carefully concerned: (*a*) prevention of loss of urine, by holding the animal over its metabolism cage, when stomach-tubing was done or injections made; (*b*) getting the blood samples injected quickly, i.e., before inactivation, and (*c*) using docile test animals under conditions of minimal disturbance. The last is best accomplished by keeping the animals in a quiet place and having the stomach-tubing done by skilled technicians. We have used test animals only once, but there is no established need for this precaution.

Controls.—The well known daily variability in diuretic response of rats to water requires that controls be run simultaneously with test animals. Controls were given injections of normal saline solution, with serum samples aged overnight and hence inactive, or of the fresh serum of hypophysectomized rats, such serum also being free from antidiuretic activity.

Type of results obtained.—Fresh serum or plasma of rats tested in this way shows an antidiuretic activity roughly approximating that of 1 mU of Pitressin. It can readily be detected that there are differences in the amounts of blood ADS in various conditions (e.g., adrenal insufficiency, in which serum antidiuretic activity is increased, or after hypophysectomy, when it is absent). The number of test animals required to reveal such differences will obviously vary with their magnitude. Usually 2 runs with 6 animals each have been made for use with statistical methods not involving variance analysis. Unfortunately, the activity of blood samples has not been carefully standardized against Pitressin, nor have adequate dose-response data been obtained. For that reason the method is spoken of here as one for the "detection" rather than for the "assay" of ADS. Some variation in the procedure, according to Dicker and Ginsburg (7), may reveal the presence of a stable antidiuretic agent which is a product of clotting; by following the method of Birnie *et al.* (5), however, Heller and Ginsburg (11) in the same laboratory have confirmed the presence of a labile ADS in normal blood. They added the significant observation that in rats, mice and sheep, jugular serum contained much more antidiuretic activity than carotid serum, as might be expected if the material was of pituitary origin. Therefore, if cardiac blood is used, that from the right side of the heart should be more potent than that from the left. Other evidence concerning the possible identity of the material in rat blood with ADH has been cited elsewhere (5, 8).

Blood of other species tested in rats.—This method will reveal variations in antidiuretic activity in the blood of various species (5, 11), including man (16, 21). Lloyd and Lobotsky (16) think, however, that there may be differences between the substances present in human and rat blood; perhaps they were dealing with the stable ADS of Dicker and Ginsburg.

Use of whole blood.—Strong evidence has been provided by the van Dyke group (1) that ADH can be detected in dog blood. They observed antidiuretic activity in 10 ml samples of whole blood drawn from the jugular veins of dogs whose osmoreceptors had been stimulated by intracarotid injections of hypertonic sodium chloride. The test animals were dogs with diabetes insipidus. Unfortunately, such test animals could hardly be used for routine clinical work.

Comment by J. Maxwell Little

Dr. Gaunt is to be congratulated on describing this procedure as a means of *detection* of antidiuretic activity rather than suggesting that it might be an assay method.

Comment by Dr. Gaunt

It is not yet an assay method, although we hope to make it one. The results cannot be expressed in unitary quantitative terms, but it can be fairly said that one blood contains more or less activity than another. And, of course, the nature of the substance(s) in blood which has this antidiuretic activity has not been established by direct means.

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ENDOGENOUS CREATININE CLEARANCE*

HENRY K. SCHOCH *and* AUGUSTO A. CÁMARA, *University of Michigan*

URINARY CREATININE of a person subsisting on a creatinine-free diet is derived solely from muscle creatine and phosphocreatine. A relatively constant amount is excreted daily; this quantity is largely a reflection of muscle mass and not of muscular activity. Exogenous creatinine begins to be secreted by the renal tubules at plasma concentrations as low as 1.8 mg per cent (10). Creatinine produced in endogenous muscle metabolism, on the other hand, is believed not to be secreted at similar plasma concentrations. Neither exogenous nor endogenous creatinine is reabsorbed by the tubules. The available evidence suggests that both are freely filtrable at the glomerulus. Thus it would seem that calculation of the renal clearance of endogenous creatinine would afford a measure of glomerular filtration rate which should closely approximate the inulin clearance. Various investigators have obtained results in normal adult subjects which tend to support this view (6, 8, 12, 15). Average endogenous creatinine/inulin clearance ratios were found to be 0.99–1.03, despite the fact that different technical methods were used.

PROCEDURE

a) Dietary preparation.—When the clearance is to be determined over a 24 hr period, the errors introduced by exogenous creatinine are largely eliminated by use of a creatinine-free diet for the 2 days immediately preceding the test as well as during the test period itself. For practical purposes, the 40 g protein diet advocated by Addis (1) for patients with renal disease is an adequate substitute. It should provide a maximal daily meat intake of 60 g. For shorter clearance periods (2–6 hr), use of a creatinine-free breakfast should suffice (2). The test should be scheduled for the morning hours immediately following the breakfast.

b) Blood specimen.—A specimen for serum creatinine concentration is obtained in the forenoon of the day of the test. In the case of the clinic patient who has completed a 24 hr collection period just prior to his return visit, a blood specimen is obtained as soon after completion of the collection as is feasible. With the dietary precautions mentioned, significant variation in the level of serum creatinine from day to day does not occur.

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* In this discussion, the term "endogenous creatinine clearance" will be used to designate the clearance of the total endogenous chromogen.

Hemolysis of the specimen or prolonged contact of red blood cells with the serum will result in falsely high values for serum creatinine concentration as the result of passage of noncreatinine chromogen from the cells to the serum. Care should be taken to use dry syringes, needles and containers and to assure gentle transfer of blood from syringe to container. Separation of serum from the clot after centrifugation should be completed within 1 hr.†

c) *Determination of serum creatinine concentration.*—The method used is a modification of that described by Bonsnes and Taussky (4). A protein-free filtrate is prepared from 3.0 ml of serum by adding 21.0 ml of distilled water, 3.0 ml of 10 per cent sodium tungstate solution and 3.0 ml of 0.66N sulfuric acid in the order listed. Thorough mixing is recommended after addition of each reagent. The mixture is allowed to stand 20 min and then is filtered through no. 1 Whatman filter paper. The filtrate appearing in the 1st 10 min is refiltered through the same paper, the collection being made in a 2d, clean test tube. This latter procedure assures a clear filtrate.

Duplicate 5.0 ml aliquots of the filtrate are transferred to colorimeter tubes,‡ and 2.0 ml of 0.04M picric acid solution is added with mixing. A blank is prepared by adding 2.0 ml of the picric acid solution to 5.0 ml of distilled water. To each colorimeter tube, including the blank, there is then added 2.0 ml. of 0.75N sodium hydroxide solution (which should be prepared with an accuracy of ±5 per cent as determined by standardization). The color which develops in each tube is then read in the colorimeter exactly 20 min after addition of the sodium hydroxide solution to the tube in question. Using a Coleman Universal or Junior spectrophotometer, a wavelength of 495 mμ gives most accurate results. (For the Evelyn photoelectric colorimeter, filter #520 is inserted and the machine set for aperture 6.)

Optical density values are compared with a standard curve showing optical density plotted against gamma of creatinine/5 ml. This curve is prepared from 5.0 ml samples of standard solutions containing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40 and 50 gamma of creatinine/5 ml, treated in the fashion described above with the fully developed color being read in exactly 20 min.

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† The use of heparinized blood and immediate centrifugation allows prompt separation of the plasma with less likelihood of hemolysis. It has the additional advantage of yielding a larger usable volume of specimen (14).

‡ For serum samples with known high creatinine content, only 3.0 or 2.0 ml (or occasionally 1.0 ml) of filtrate is used. Distilled water is added in accurately measured amounts such as to produce a volume of 5.0 ml before addition of the picric acid and sodium hydroxide solutions. Appropriate correction for this dilution is made in calculating the serum concentration.

In our experience such a standard curve remains stable for several years as determined by frequent interval checking with standard solutions. The Jaffe color does not adhere to Beer's law, and it is important to dilute the serum (and urine) specimens such that their optical density values will fall on that portion of the curve which most closely approximates Beer's law. In the standard curve described here, this corresponds to the range of 5–20 gamma of creatinine/5 ml. Sirota (14) has observed definite changes in the standard curve with different environmental temperatures and occasionally with a newly standardized sodium hydroxide solution. As a consequence, he routinely uses 5 standard solutions with each series of determinations and dilutes urines and plasmas such that the expected values fall somewhere on the 1st half of the curve, but below 85 per cent transmission. When the Bonsnes-Taussky proportions are used, the color is stable for 15 min after full color development, as noted by Sirota (14), and a delay in reading of up to 15 min will thus not invalidate the observations.

d) Urine specimen.—If a 24 hr clearance is desired, urine is collected by spontaneous voiding over an accurately timed 24 hr period, the specimen being preserved with 10 ml. of toluene. Analysis should be performed promptly following completion of the collection. For shorter periods, spontaneous voiding may be utilized for the collection in subjects under 50 years provided there is nothing to suggest retention of urine in the bladder. Those past 50 or those in whom retention is suspected should be catheterized after spontaneous voiding to ascertain whether or not residual urine is present. If so, collection of specimens should be by catheterization.

e) Determination of urine creatinine concentration.—Dilutions of the urine are made such that the creatinine content of 5.0 ml of the diluted urine may be expected to fall in the range of 10–30 gamma. The dilution commonly used is 1:200 or 1:250. Knowledge of past specimens from the individual patient aids in judging the proper dilution, since the 24 hr excretion of creatinine (or the excretion over shorter periods, under standard conditions) tends to be relatively constant. The diluted urine is processed just as the serum filtrate, using 5.0 ml aliquots in duplicate. The same standard curve is used in calculating urine concentration of creatinine, and from this the total urinary content of creatinine is obtained.

f) Calculation of clearances.—In the case of the 24 hr endogenous creatinine clearance, the calculation (corrected to a standard surface area of 1.73 sq m) is as follows:

$$\text{Corrected clearance (in l/24 hr)} = \frac{\text{24 hr urinary excretion (mg)}}{\text{serum creatinine concen. (in mg/l)}} \times \frac{1.73}{\text{observed surface area (in sq m)}}$$

If shorter collection periods are used, the calculations are made with a similar formula substituting the urinary excretion in mg/min for total 24 hr excretion and the serum level in mg/ml for mg/l, the final clearance being expressed as ml/min rather than as l/24 hr.

Normal values.—We have routinely employed the 24 hr determination of clearance for reasons discussed elsewhere (7). After 2 days on a creatinine-free diet, the normal values for both males and females, corrected to a standard surface area of 1.73 sq m, average 137 l/24 hr. The normal range is approximately 85–115 per cent of this figure.

Sirota, Baldwin and Villarreal (13) found a mean value of 111 ml/min for normal males on an unrestricted diet, utilizing a 4 hr collection period (8:00 A.M. to noon). Addis and co-workers (2), utilizing a similar collection period (7:00 A.M. to noon), obtained a mean value of 122 ml/min for normal males who had eaten a creatinine-free breakfast. The latter value is corrected to a standard body weight rather than to a standard surface area.

Limitations.—The unanswered problems regarding the nature of the various plasma chromogen substances which yield color with alkaline picrate place a serious limitation on the routine use of the endogenous creatinine clearance in studies which require accurate measurement of glomerular filtration rate. Some 75 per cent or more of the total plasma chromogen represents true creatinine in normal subjects (9, 11), while less than 5 per cent of urinary chromogen is other than true creatinine. Increase in noncreatinine chromogen content of the plasma, as occurs in cardiac disease (3, 13), will give falsely low clearances. Tubular excretion of creatinine, on the other hand, is known to occur in renal disease (5, 6) and tends to give clearance values significantly higher than the true glomerular filtration rate. This test cannot, then, be considered a valid investigational technique in normal subjects or in individuals with disease, with the possible exception of the circumstance in which sufficient determinations of simultaneous inulin and endogenous creatinine clearances in a given individual permit calculation of filtration rate from the creatinine clearance.

Applications.—As a *clinical* measure of glomerular filtration rate, however, the endogenous creatinine clearance is distinctly superior to either the inulin or the urea clearance. Diurnal variation and the minor alterations in glomerular filtration rate which result from changes in posture, variation in intra-abdominal pressure or the intake of food need not be considered when collections are made over a complete metabolic time period such as the 24 hr cycle (7). Reproducibility of results is surprisingly good, the limit of variability in 24 hr clearances usually being less than 5 per cent. The fact that the endogenous creatinine clearance overestimates glomerular filtration rate by as much as 10 per cent when renal function is reduced to $^1/_3$ of normal (6), and by a progressively greater amount with further decrease in function, need not invalidate the determination as a useful method whereby the course of the patient's disease may be observed. In actuality, the absolute differences between true glomerular filtration rate and endogenous creatinine clearance, even in patients with advanced renal disease, are so slight as to possess little clinical significance.

Note.—This section was reviewed by Jonas H. Sirota.

Comment by A. C. Corcoran

This article is intended for the attention of those in clinical practice who find that urea clearance is not a satisfactory index of glomerular filtration rate under the conditions in which these measurements are so often made. Where such is the case, an alternative procedure which concentrates its pitfalls in the laboratory and in the economy of the body rather than in the ward and ward attendant would be welcome. The author and the reviewer have alike had wide experience with the clinical use of endogenous creatinine clearance and they conservatively assess its value.

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MICROBIOLOGIC ESTIMATION OF ARGININE Tm IN THE DOG

KARL H. BEYER *and* LEMUEL D. WRIGHT, *Sharp and Dohme, Inc., West Point, Pa.*

Renal tubular participation in the elaboration of urine consists in modifying glomerular ultrafiltrate by a variety of secretory and reabsorptive processes. The energy available for the enzymatic mechanisms on which these functions depend seems to be allocated and committed, as it were by quanta, to functions some of which are highly specific and some, e.g., growth and repair more general (1). Grouping of functional and metabolic processes is evidenced by the mutual competition of some compounds (e.g., PAH and penicillin) for secretory mechanisms (2) and others (glucose and xylose, arginine and lysine) for reabsorptive pathways (2, 4, 13). The energy requirements of other processes are seemingly independent, as indicated by the lack of competition for concurrent secretion of PAH and N^1-methylnicotinamide or of competition for reabsorption between essential monoamino and diamino acids (3, 4).

Renal disease, renotropic agents and nephrotoxins may differentially alter specific functions. With recognition of selective alterations of functions has come the development of methods that yield specific information relating to maximal tubular secretory capacity (Diodrast, PAH) or reabsorptive capacity (glucose). Measurement of renal tubular reabsorptive capacity for arginine and lysine can contribute to a still more precise analysis of tubular disease and malfunction. The reabsorptive pathways for these 2 amino acids are at least in part the same, as shown by mutual competition for reabsorption. Further, their reabsorptive maxima are better defined than those of most other amino acids (5, 11, 20).

Of these 2 amino acids, natural arginine is commercially available at a reasonable cost. Its microbiologic determination in body fluids is not difficult. Microbiologic assays are more specific than determinations based on detection of amino or carboxyl groups. Since arginine is substantially nontoxic when administered in large doses to dogs, determination of its maximal rate of reabsorption (Tm) can be applied to the study of aberrations of tubular function which result in hyperaminoaciduria whether of congenital, renotropic or other origin.

As in the estimation of most other reabsorptive maxima, it is assumed that arginine is not secreted by the tubules and that Tm represents the difference between the amount of the amino acid that is presented to the tubules in glomerular filtrate and that which is concurrently excreted in the urine. The plasma concentrations must be sufficiently high during Tm determinations that the reabsorptive capacity (Tm) and not the load ($GF \cdot P$) presented for reabsorption limits that rate. An arginine plasma concentration of about 20 mg/100 ml (200 γ /ml) has seemed satisfactory for this purpose.

The maximal reabsorptive capacity for arginine may be expressed as a formula

$$\text{Tm (mg/min)} = (P \cdot GF) - UV$$

where P = plasma concentration of arginine in mg/ml; GF = creatinine clearance or glomerular filtration rate in ml/min; UV = amount of arginine excreted/min, where U = arginine concentration in urine in mg/ml and V = urine flow in ml/min.

TABLE 1.—PROTOCOL FOR ARGININE TM ESTIMATION

Dog 370, 12.4 kg, surf. area = 0.60 sq m, Exper. 9196

Time, Hr: Min	Elapsed, Min	Sample	Urine Vol, Ml[*]	Wash-out Dil.	Vol, Ml/-Min	GF[†]/V	Approx. Dil.[‡]	Actual Dil.[§]
	Postabsorptive condition. Fasted overnight.							
0:00	650 ml H_2O by stomach tube							
1:00	400 ml H_2O by stomach tube							
1:25	L(+)-arginine venoclysis begun; 5 mg/kg/min in normal saline at 3 ml/min[‖]							
1:26	Creatinine 2.5 g subcutaneously (25 ml of 10% sol.)							
1:30	Arginine intravenous single "priming dose" = 3.0 mg/kg (2 ml of 1.86% sol.)							
1:45	0	U_0						
1:51	6	P_1						
1:55	10	U_1	49	1.31	4.9	12.24	9.0	23.58
2:05	5	P_2						
2:10	10	U_2	49	1.31	4.9	12.24	9.0	23.58

[*] Including 15 ml of sterile water for bladder wash-out, which was followed by insufflation of the bladder with air to assure complete emptying.

[†] Estimated glomerular filtration rate (GF) = 60 ml/min.

[‡] 12.24/1.31 = 9.34 or 9.0. This figure is rounded off (9.0) for analytical convenience.

[§] 9 × 1.31 × 2 (since 0.5 ml urine was used according to the procedure outlined) = 23.58. This is the actual dilution used to convert mg of creatinine/tube to mg/100 ml in Table 2. These dilutions were made so that urine concentrations would approach plasma concentrations for analysis.

[‖] 200 ml of 1.86% arginine in normal saline is sufficient for the "priming dose" and venoclysis for this experiment using a 12.4 kg dog.

PROCEDURE

Determination of renal clearance.—A protocol for determination of renal clearances from which the arginine Tm is calculated is presented in Table 1. Table 2 summarizes the calculation of creatinine and arginine clearances and the final expression of Tm.

In general, dogs are fasted overnight in order to avoid the dynamic action of foodstuff and to enhance the absorption of materials administered by gavage. The animals are given sufficient water to assure adequate urine flow in order to minimize errors due to retention of small amounts of urine or sterile water with which the bladder is washed. The "priming" and venoclysis of arginine are to attain quickly and to maintain the high plasma concentrations of arginine required to present to the tubules an ultrafiltrate of sufficient concentration to "saturate" the reabsorptive mechanism involved. The purpose of the series of dilution calculations is to reduce the concentration of creatinine in urine (due to reabsorption of water) to where the same analytical procedure described for plasma (alkaline picrate method) can be applied. Since the amount of arginine reabsorbed per unit time is the difference

TABLE 2.—CALCULATION OF CREATININE CLEARANCE AND ARGININE TM

Dog 370, 12.4 kg, surf. area = 0.60 sq m, Exper. 9196

Creatinine

SAMPLE	GALV. READING DUPLICATE	GALV. READING AV.	MG/TUBE	DIL.*	MG/100 ML	U/P RATIO	V, ML/MIN	UV/P, ML/MIN
P_1	30^2 30^2	30^2	0.0850	200	17.0	11.31	4.9	55.4
P_2	30^2 30^2	30^2	0.0850	200	17.0	11.72	4.9	57.4
U_1	32^0 31^3	31^3	0.0815	23.58	192.2			
U_2	28^1 27^1	27^3	0.0930	23.58	219.3			

Arginine

SAMPLE	γ/ML†	MG/100 ML	U/P RATIO	V, ML/MIN	UV/P, ML/MIN	AMT FILTERED,‡ MG/MIN	UV, MG/MIN	TM,§ MG/MIN/SQ M
P_1	198	19.8	1.76	4.9	8.6	10.96	1.71	15.5
P_2	228	22.8	2.98	4.9	14.7	13.08	3.34	16.1
U_1	349	34.9						
U_2	683	68.3						

* As indicated in the procedure B(*b*), since these data were procured an additional 2-fold dilution has been introduced to increase the galvanometer readings, thus bringing most of them into a more critical part of the standard curve.

† The system of dilutions used for creatinine is inappropriate for arginine. The γ/ml value for arginine is calculated on the basis of the assay presented in Table 3.

‡ Arginine plasma concentration in mg/ml × glomerular filtration rate in ml/min.

§ $\text{Tm} = \frac{(\text{Amt filtered} - UV)}{0.60}$. This has been corrected to a surface area of 1.0 sq m by dividing actual amount reabsorbed by actual surface area of the dog (0.60 sq m).

between the amount filtered and that which is excreted, it is necessary to determine the simultaneous glomerular filtration rate which is equivalent to creatinine clearance in the dog (10, 14) and to mannitol (18) or inulin clearance in man (16).

Creatinine determinations.—These are carried out on a protein-free filtrate prepared by the alkaline picrate method of Folin and Wu (7).

A. METHOD: *a*) Preparation of protein-free plasma filtrate: mix 1.0 ml plasma, 0.5 ml 0.67N sulfuric acid, 0.5 ml 10 per cent sodium tungstate and 18 ml distilled water; let stand for 10 min and filter.

b) Dilution of urine for analysis:

$$\frac{\text{estimated clearance (ml/min)}}{\text{urine vol (ml/min)} \times \text{wash-out dilution}}$$

c) Preparation of alkaline picrate solution: *immediately before use*, mix 1.0 ml 10 per cent sodium hydroxide, 5.0 ml saturated picric acid and 6 ml distilled water.

d) Pipet 5.0 ml plasma filtrate from (*a*) or 0.5 ml diluted urine plus 4.5 ml distilled water (*b*) into colorimeter tube. Add 10 ml of alkaline picrate solution (*c*) and let stand 15 min. Read in an Evelyn colorimeter using a #520 filter, 10 ml aperture against a blank tube containing 5.0 ml distilled water and 10 ml alkaline picrate solution.

B. CALCULATIONS: *a*) Plasma (mg/100 ml) = mg/tube (read from curve) × 400

b) Urine (mg/ml) = mg/tube (read from curve) × 2 × wash-out × urine dilution

c) $$\text{Clearance (ml/min)} = \frac{\text{urine conc.} \times \text{urine vol}}{\text{plasma conc.}}$$

With an Evelyn photoelectric colorimeter using a #520 filter, creatinine concentration in plasma and urine is conveniently estimated. The galvanometer deflections for the various samples are read against a reagent blank set at 100 (air blank = 88°) and compared to a standard curve for estimation of absolute values. Creatinine data corresponding to the protocol of the experiment given in Table 1 are set forth in Table 2.

Arginine determination.—This is performed on protein-free filtrates prepared with tungstic acid according to the method of Dunn *et al.* (6). For the tungstate precipitation of plasma, mix 2 ml plasma, 8 ml water, 1 ml $^2/_3$ N sulfuric acid and 1 ml of 10 per cent sodium tungstate; let stand for 2 min, centrifuge, then neutralize the centrifugate.

The microbiologic estimation of arginine according to the method of Stokes *et al.* (18), using his basal medium and *Strepto-*

coccus faecalis R as the organism, is recommended. Stoke's basal medium contains:

DL-leucine	100 mg
DL-iso-leucine	100 mg
DL-valine	100 mg
L(−)-cystine	100 mg
DL-methionine	100 mg
DL-tryptophane	200 mg
L(−)-tyrosine	100 mg
DL-phenylalanine	100 mg
DL-glutamic acid	100 mg
DL-threonine	100 mg
DL-alanine	100 mg
DL-aspartic acid	100 mg
L(+)-lysine	50 mg
L(+)-histidine	100 mg
DL-serine	100 mg
L(−)-proline	100 mg
L(−)-hydroxyproline	100 mg
DL-nor-leucine	100 mg
glycine	100 mg
glucose	5 g

sodium acetate (anhydrous)	3 g
adenine	5 mg
uracil	5 mg
guanine	5 mg
pantothenic acid	100 γ
riboflavin	100 γ
thiamine hydrochloride	100 γ
nicotinic acid	100 γ
pyridoxamine	200 γ
p-aminobenzoic acid	20 γ
biotin	0.1 γ
folic acid	1.0 γ
salts A	
K_2HPO_4	250 mg
KH_2PO_4	250 mg
salts B	
$MgSO_4 \cdot 7H_2O$	100 mg
NaCl	5 mg
$FeSO_4 \cdot 7H_2O$	5 mg
$MnSO_4 \cdot 4H_2O$	5 mg

Adjust to pH 6.8 and add distilled water to 250 ml.

TABLE 3.—ASSAY PROTOCOL FOR MICROBIOLOGIC ESTIMATION OF ARGININE*

TUBE	STANDARD CURVE		UNKNOWN SAMPLES	
	STOCK SOL., ML	FINAL CONC. L(+)-ARGININE, γ/10 ML CULTURE	PROTEIN-FREE PLASMA FILTRATE, ML OF DIL.	URINE, ML OF DIL.
1	0	0		
2	0	0		
3	0.5	10		
4	1.0	20		
5	1.5	30		
6	2.0	40		
7	3.0	60		
8	5.0	100		
9			0.5	
10			1.0	
11			2.0	
12			4.0	
13				1
14				2
15				3
16				5

* Five ml of double strength medium is added to all tubes. After autoclaving at 120 C for 15 min, all tubes were inoculated with 1 drop of a suspension of *Str. faecalis R* cells obtained by centrifugation of a 24 hr culture and resuspension of the cells in 10 times the original volume of sterile saline.

A standard curve for L(+)-arginine is set up with each experiment as indicated in Table 3. After 18 hr of incubation at 37 C, turbidimetric estimations of growth are performed using a Klett-Summerson photoelectric colorimeter, a #54 filter and a galvanometer zero setting against water as a reference medium. The amounts of arginine present in plasma and urine are estimated by comparison of galvanometer readings of the unknown samples with the standard curve run concurrently. Calculations of arginine clearance and Tm values for the experiment presented in Table 1 are summarized in Table 2.

Comment.—Russo *et al.* (12) analyzed the result of arginine Tm values of dogs employed in clearance studies in this laboratory over the years 1946, 1947 and 1949. It was found that the difference between Tm values was not significant but that the mean arginine Tm for 1949 was lower than that for 1946 and 1947. Even so, the mean value and standard of a single determination for any dog for any one occasion was 12.9 ± 1.7 mg/min/sq m of surface area. The 95 per cent confidence limits were 12.9 ± 3.3 mg/min/sq m (8).

TABLE 4.—Reproducibility of Arginine Tm on Different Days
Dog 370, 12.4 kg, surf. area = 0.60 sq m

Clearance Period	Urine Flow, Ml/Min	Creatinine Clearance, Ml/Min	Arginine	
			Clearance, Ml/Min	Tm, Mg/Min/Sq M
1st Exper.				
1	4.9	55.4	8.6	15.5
2	4.9	57.4	14.7	16.1
2d Exper.				
1	4.5	44.6	11.8	15.1
2	3.7	44.0	13.1	16.0

Table 4 illustrates the reproducibility of successive Tm values based on 10 min clearance periods. The constancy of arginine Tm determinations on different days for the same animal is shown in Table 4 by the data from 2 experiments performed several days apart on the same dog. This reproducibility of arginine Tm obtained despite a considerable variation in glomerular filtration on the 2 occasions.

We have used the microbiologic estimations of amino acids for clearance determinations because of their greater specificity. Pitts first described the Tm for arginine using the more conventional ninhydrin reaction, which reacts in general with alpha-amino acids (9). However, under conditions where the values for arginine Tm are to be given greatest interpretive weight, the microbiologic estimation inherently should be the most critical. In the hands of experienced laboratory workers, quantitative microbiological assays are as reliable as are chemical determinations.

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IN VITRO METHODS FOR STUDY OF RENAL TUBULAR EXCRETION

JOHN V. TAGGART, *Columbia University, and* ROY P. FORSTER, *Dartmouth College*

A GROWING interest in the metabolic aspects of cellular transport has led to the development of a variety of in vitro techniques which are particularly useful in studying renal transport mechanisms. The procedures to be described have certain features in common and similar advantages and disadvantages. They represent outgrowths of the method introduced by Robert Chambers and his co-workers in which the accumulation of phenol red was observed in tissue-cultured cystic explants of embryo chick mesonephros.

The absence of glomerular filtration in any in vitro preparation of kidney limits observations to those on tubular excretion; no satisfactory methods exist for studying tubular reabsorption in vitro. Certain of the excretory mechanisms are known to be active processes capable of establishing large concentration gradients across the tubular epithelium. Consequently, satisfactory quantitative results can be obtained in vitro with small tissue samples.

The in vitro techniques are not intended to replace in any sense the well established experimental procedures for renal studies in the intact animal. Their principal usefulness lies in facilitating biochemical observations on transport mechanisms. The composition of the ambient fluid can be rigidly controlled and can be varied over a much wider range than is possible in the intact animal. Various chemical agents and enzyme inhibitors, too toxic for use in the intact animal, can be examined for their specific effects on selected renal mechanisms. The extrarenal effects of these agents which may alter kidney function indirectly are excluded. Finally, the technical simplicity of the procedures makes possible a much larger number of experimental observations than can be conveniently obtained by the clearance technique.

I. Phenol Red Transport in Isolated Fish Tubules and Frog Kidney Slices

Long segments of renal tubules can be readily isolated from the kidneys of many marine and fresh water fishes. Fish kidneys are particularly suitable because a very limited intertubular cement substance permits the separation of individual tubules. Satisfactory preparations have been obtained from the common winter flounder (*Pseudopleuronectes americanus*), killifish (*Fundulus heteroclitus*), sculpin (*Myoxocephalus octodecimspinosus*) and the fresh water hornpout, catfish or bullhead (*Ameiurus nebulosus*). The flounder tubule is apparently undifferentiated, whereas that of Ameiurus contains readily distinguishable proximal and distal convoluted segments.

When isolated fish tubules are incubated in a dilute solution of phenolsulfonphthalein (phenol red) in a balanced saline medium, concentration of the dye is apparent within the tubular lumina in 5–10 min and increases in 30–60 min to approximately 100 times that in the medium. The principal advantage of the isolated tubule preparation over Chamber's technique is in obviating the use of tissue culture methods.

PROCEDURE

1. *Isolated fish tubules.*—Observations should be made only on freshly caught live specimens. The abdominal cavity is cut open and the kidney transferred to a beaker of the saline medium (without phenol red). Fragments approximately 1 mm across are teased from the kidney with fine forceps and placed in individual Petri dishes containing 5 cc of a freshly prepared and actively oxygenated medium of the following composition:

FOR MARINE FISHES: NaCl 0.783 per cent (134 mM), KCl 0.0186 per cent (2.5 mM), $CaCl_2$ 0.0167 per cent (1.5 mM), $MgCl_2$ 0.00953 per cent (1.0 mM), $NaH_2PO_4 \cdot H_2O$ 0.0069 per cent (0.5 mM), $NaHCO_3$ 0.126 per cent (15.0 mM), and phenol red 0.001 per cent (0.028 mM).

FOR FRESH WATER FISHES: Same as for marine fishes, except that NaCl is reduced to 0.585 per cent (100 mM) and $NaHCO_3$ to 0.042 per cent (5.0 mM).

The pH of the medium is approximately 8.4, but satisfactory phenol red accumulation can be observed within the pH range of 7.0–8.6. Although bicarbonate is not an essential component, the alkalinity which it imparts to the medium insures the red color of the dye and facilitates quantitative comparisons. Experiments are performed at room temperature. Continuous oxygenation is supplied to each Petri dish through a 2 in. 22 gauge hypodermic needle bent to fit over the rim. The initial teasing, together with the gentle agitation provided by the stream of oxygen bubbles, quickly separates the individual tubules. From time to time the Petri dish containing the kidney fragments is placed on a microscope stage for examination with 50–100× magnification. The concentration of phenol red in the lumina is graded as follows: 0 indicates no accumulation, + a barely detectable concentration (5-fold), and ++ to ++++ successive increases to approximately 100-fold. Since all the tubules do not accumulate dye at an identical rate, the sample is graded on the basis of the most active segment observed.

Metabolic intermediates and inhibitors are dissolved in the medium and the pH is readjusted to 8.4. In the usual experiment, 7 Petri dishes are run simultaneously; 1 serves as the control and the remaining dishes contain various concentrations of the agent under study. Experiments are considered satisfactory only when the control shows ++++ concentration of dye within 30–60 min.

2. *Frog kidney slices.*—Frog kidney can be sliced freehand with a razor blade into thin longitudinal sections which are satisfactory for studying phenol red transport. The incubation medium is the same as that used for fresh water fishes except that 0.5 per cent glucose is added. A natural yellow pigmentation of frog kidney slices makes the interpretation of dye concentration somewhat more difficult than in the isolated tubule preparations.

II. Phenol Red Transport in Guinea Pig Kidney Slices

Beyer and his associates (2) have modified the method of Forster (6) to permit the study of phenol red transport in slices of mammalian kidney. Guinea pig renal cortex is apparently the most satisfactory, but qualitatively similar results have been obtained with rabbit, mouse and rat kidney. In slices of mammalian kidney, phenol red transport can be shown to occur only in the proximal convoluted tubules.

PROCEDURE

A guinea pig is exsanguinated and the kidneys are removed to a freshly prepared and chilled modified Krebs-Ringer bicarbonate solution of the following composition: NaCl 0.85 per cent (144 mM), KCl 0.043 per cent (5.75 mM), KH_2PO_4 0.019 per cent (1.44 mM), $MgSO_4 \cdot 7H_2O$ 0.036 per cent (1.44 mM), and $NaHCO_3$ 0.403 per cent (48 mM). Thin slices of kidney cortex are sectioned freehand with a razor blade and incubated in 5 cc of medium through which 5 per cent CO_2–95 per cent O_2 is bubbled continuously. The final pH of the medium is 7.8. The temperature during incubation is maintained at 37 C in a thermostatically controlled glass chamber. After 5 min of incubation, phenol red is added to the medium in a final concentration of 4 mg per cent. After an additional 15–30 min the slices are removed, spread on a glass slide and examined immediately under the microscope at 30–60× magnification. The accumulated dye appears as streaks of red clearly outlining the tubular lumina.

Studies in Warburg apparatus.—Studies with volatile inhibitors (e.g., HCN) can be performed in the Warburg apparatus. Beyer recommends that the tissues be exposed to inhibitors, particularly cyanide, for several minutes before addition of phenol red. Consequently, the kidney slices and medium are added to the main compartment of the vessel and the phenol red is tipped in from a side-arm after a 5 min equilibration period. The gas space is filled with 5 per cent CO_2–95 per cent O_2. The slices are removed from the vessel after 15 min and examined in the usual manner. When measurements of respiration are desired, the medium is modified by replacement of $NaHCO_3$ by an equivalent amount of sodium phosphate buffer of pH 7.8, the CO_2–O_2 mixture is replaced by 100 per cent O_2, and alkali is placed in the center well of the Warburg vessel.

III. Transport of PAH, Diodrast and Phenol Red in Rabbit Kidney Slices

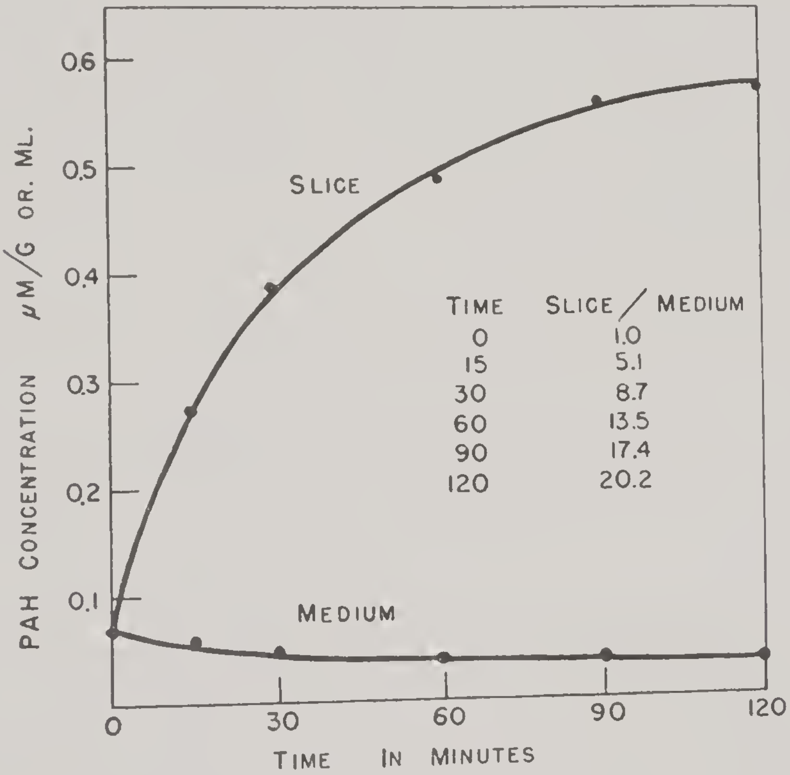
The slice technique (6) has been modified by Cross and Taggart (4) to permit quantitative studies on the tubular transport of compounds other than phenol red.

PROCEDURE

Experiments are performed in the conventional Warburg apparatus. For studies on *PAH transport*, the main chamber of each vessel generally contains 1.18 cc of 0.3M NaCl, 0.27 cc of 0.1M KCl, 0.3 cc of 0.1M Na acetate, 0.2 cc of 0.1M Na phosphate buffer of pH 7.4, 0.1 cc of 0.02M $CaCl_2$, 0.2 cc of 0.001M Na PAH, and water to a final volume of 2.7 cc. When other compounds requiring neutralization are used, they are added as the sodium salts and the NaCl is reduced by an equivalent amount. The loaded vessels are chilled in crushed ice.

A rabbit is exsanguinated and the kidneys are promptly transferred to a beaker of ice-cold 0.13M NaCl–0.02M KCl. Slices of renal cortex 0.3–0.4 mm thick are cut in a Stadie-Riggs microtome (9). After brief blotting on filter paper, the slices are weighed on a torsion balance and approximately 300 mg is added to each vessel. Filter paper and 0.2 cc of 6N NaOH are placed in the center well, the cups are mounted on manometers, and the gas space is filled with oxygen. The vessels are then shaken in a 25 C water bath at a rate of 100 c/min. After 15 min equilibration, the taps are closed and oxygen consumption is recorded during the following 30 min. Oxygen uptake is expressed as a QO_2 (cu mm of O_2 consumed/mg initial wet weight of tissue/hr).

After 1 hr in the bath, the vessels are removed and promptly chilled in crushed ice. The slices are removed as soon as possible, blotted carefully on filter paper, weighed and transferred to a graduated cylinder to which are added 3 cc of 10 per cent trichloroacetic acid and water to a final volume of 10 cc. A 2 cc aliquot of the final suspending medium is treated with trichloroacetic acid in a similar manner. The solutions are filtered after 20 min and 5 cc aliquots of the filtrates are used for estimation of PAH by the method of Bratton and Marshall (3). The accumulation of PAH in kidney slices is expressed as the ratio of PAH concentration in slices/medium (S/M), as shown in Figure 1. Recoveries of added PAH average about 98 per cent.



TIME	SLICE / MEDIUM
0	1.0
15	5.1
30	8.7
60	13.5
90	17.4
120	20.2

FIG. 1.—Accumulation of PAH by rabbit kidney slices.

The rate of PAH accumulation in rabbit kidney slices is greatly enhanced by presence of acetate in the suspending medium (4). Other oxidizable substrates, such as members of the citric acid cycle, certain amino acids and the longer chain fatty acids, are strongly inhibitory in concentrations as low as 0.01M.

For studies on *Diodrast transport*, the commercial product is converted to the sodium salt by crystallization of the free acid and subsequent neutralization with NaOH. Each vessel contains 0.2 cc of 0.0075M Na Diodrast in place of PAH. Cadmium filtrates (8) of the slices and medium are used. The slices should be allowed to extract in the $CdSO_4$–H_2SO_4 solution for 20 min before the addition of NaOH. Diodrast is estimated by the iodometric method of Alpert (1).

Quantitative studies with *phenol red* are somewhat less satisfactory in that the dye accumulated in the slices cannot be completely recovered. The final concentration of phenol red in the medium is estimated directly by diluting an aliquot in 0.01N NaOH and measuring the optical density at 550 mμ. The total quantity and concentration in the slices are calculated on the basis of the difference between the amount of phenol red added and that recovered in the medium.

NOTE.—This section was reviewed by Karl H. Beyer.

Comment by A. C. Corcoran

The techniques here described provide a novel, dramatic and undoubtedly informative approach to the study of the mechanisms of tubular transfer.

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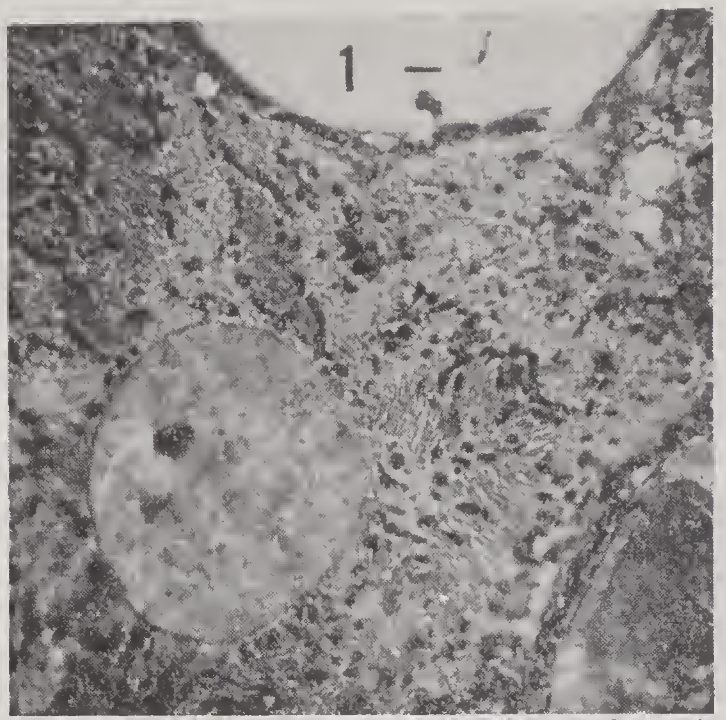
PREPARATION OF TISSUE SECTIONS FOR ELECTRON MICROSCOPY

A. J. DALTON, *National Cancer Institute*

EARLY EFFORTS in the development of methods of obtaining ultrathin sections for observation of tissues with the electron microscope included use of wedge sections (25) and development of the high-speed microtome (11, 20). These efforts were moderately successful and certainly demonstrated the feasibility of thin sectioning for electron microscopy but involved a considerable element of chance. The highspeed microtome remains a useful mechanism for obtaining thin sections of excessively hard substances, but the adaptation of various models of microtomes normally used for routine histologic sectioning for use in ultrathin sectioning (1, 2, 9, 10, 14, 18, 23) and introduction of the glass knife (16) has made tissue sectioning for electron microscopy practically a routine procedure in many laboratories. Although in this laboratory our experience has been confined almost exclusively to the thermal expansion method of sectioning following the n-butyl methacrylate embedding method (18), it has been demonstrated that with proper use of any one of these methods, sections satisfactory for electron micrography may be obtained (2, 9, 10, 18, 23). Thus the problem of thin sectioning, which many investigators undoubtedly felt would take many years to solve, is no longer a barrier to progress in the field.

Fixation of tissues for electron microscopy, which until recently received but little attention, now looms as a major and complex problem, relatively difficult of solution. The criteria of classic cytology for determining the adequacy of fixation are of some aid in this respect, e.g., comparison of structures in fixed preparations, such as nucleoli, nuclear membrane, mitochondria and cell membrane, with their counterparts in living cells. Yet it is well known that materials apparently satisfying these criteria reasonably well under the light microscope, reveal relatively gross artefacts when examined with the electron microscope. There is, in addition, the obvious problem of how to determine the degree of effectiveness of preservation of structures too small to be resolved with the light microscope. In the absence of any standard of comparison or reference, a reasonable approach would appear to be that of assuming tentatively that a process of fixation which preserves microscopic structures adequately on exposure to the electron beam also pre-



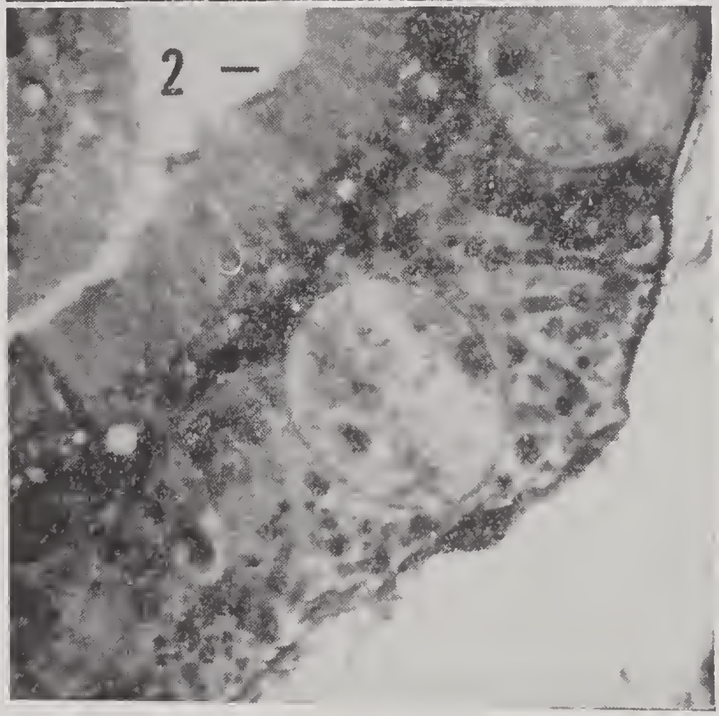


Fig. 1.—Portion of a hepatic cell of a mouse illustrating results obtained following fixation by perfusion with the osmic, dichromate, lanthanum mixture. Nuclear detail is reasonably good and mitochondria and submicroscopic lamellae are sharply delineated in the cytoplasm. Approximately 4,800×. (In Figs. 1–5, the horizontal line is the equivalent of 1 μ.)

Fig. 2.—Cell of straight portion of a proximal tubule of a mouse kidney fixed as the cell in Figure 1. Nuclear contents, nuclear membrane and mitochondria show little evidence of distortion. Approximately 4,800×.

serves submicroscopic structures satisfactorily under the same conditions.

It has long been known that, with direct contact, osmic acid in solution or in vapor form fixes and preserves cytoplasmic components more quickly and in more nearly the form and distribution they possess in living cells than any other of the fixatives of classic cytology (28). For electron microscopy the added advantages of osmic acid as a fixative are its ability to combine chemically with certain cellular constituents, thus increasing contrast, and its capacity to harden tissues to an extent which reduces the possibility of introduction of secondary artefact during the later processes of dehydration, embedding and sectioning. Some of the disadvantages of its use are its poor penetrating power (approximately 0.5 mm/hr through parenchymatous tissues), its tendency to cause electron scattering either as a result of its precipitation within the nucleus in a reduced form or because of its chemical combination with nuclear components, and, after somewhat longer exposure to it and possibly for the same reasons, loss of detail in the cytoplasm also. Fixation by perfusion circumvents the 1st of these disadvantages, and the addition of lanthanum nitrate to precipitate nucleic acids and of potassium dichromate to reduce the deposition of osmium in the tissues largely eliminates the latter 2. Thus in mice anesthetized by intraperitoneal injection of 1 cc of 0.2 per cent Nembutal containing 0.1 per cent sodium nitrite or sodium nitroprusside, perfusion with approximately 10 cc of cold 0.85 per cent sodium chloride followed immediately by perfusion with a mixture of equal parts of 2 per cent osmic acid, 3 per cent potassium dichromate and 2 per cent lanthanum nitrate, followed by immersion of thin slices of the perfused tissue in the same mixture for 24 hr resulted in satisfactory fixation and preservation of both cytoplasmic and nuclear detail in hepatic cells (7) (Fig. 1), in the kidney (5) (Figs. 2 and 3) and in the intestine and pancreas (6). For tissues containing large amounts of lipid, e.g., tissues of the nervous system, perfusion with the more concentrated solution of 2 per cent osmic acid, 3 per cent potassium dichromate and 2 per cent lanthanum nitrate, by weight, followed by immersion of thin slices in the same solution for 4–8 hr has been more satisfactory.

In general, whatever the fixative used, fixation by perfusion has given consistently better results than fixation by immersion. However, for biopsy material and for other material which for technical reasons cannot be perfused, fixation by immersion of thin slices of the tissue in Regaud's fluid for 3 days, followed by mordanting in 5 per cent potassium dichromate at 37 C for 3 days, followed by treatment with 2 per cent osmic acid at room temperature for 4–24 hr has given reasonably satisfactory results (see Fig. 4).

For visualization of the Golgi substance, perfusion with modified Champy fluid (equal parts of 2 per cent osmic acid, 3 per cent potassium dichromate and 1 per cent chromic acid), immersion of thin slices in the same mixture for 24 hr and vigorous washing in tap water for 24 hr and treatment with 2 per cent osmic acid at 37 C for 3 days has given excellent results (Fig. 5).

It is hoped and fully expected that greater emphasis on and recognition of the primary importance of fixation of tissues for electron microscopy will, in the future, bring forth more satisfactory methods of fixation than those described here. These methods have, in our hands, given more satisfactory results than methods previously described in the literature, e.g., perfusion with osmic acid alone (3), fixation by immersion in osmic acid (24) or Formalin (26) or fixation by immersion in a series of certain chemical agents (12, 13).

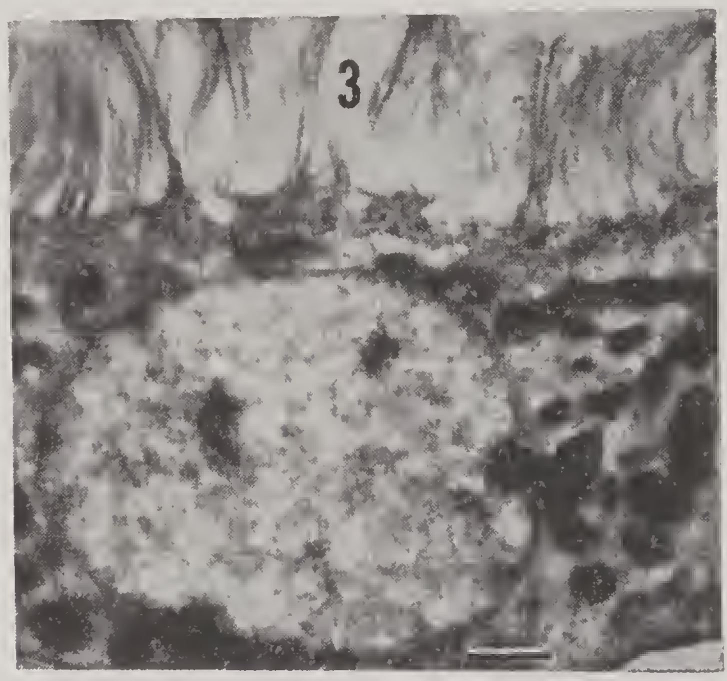
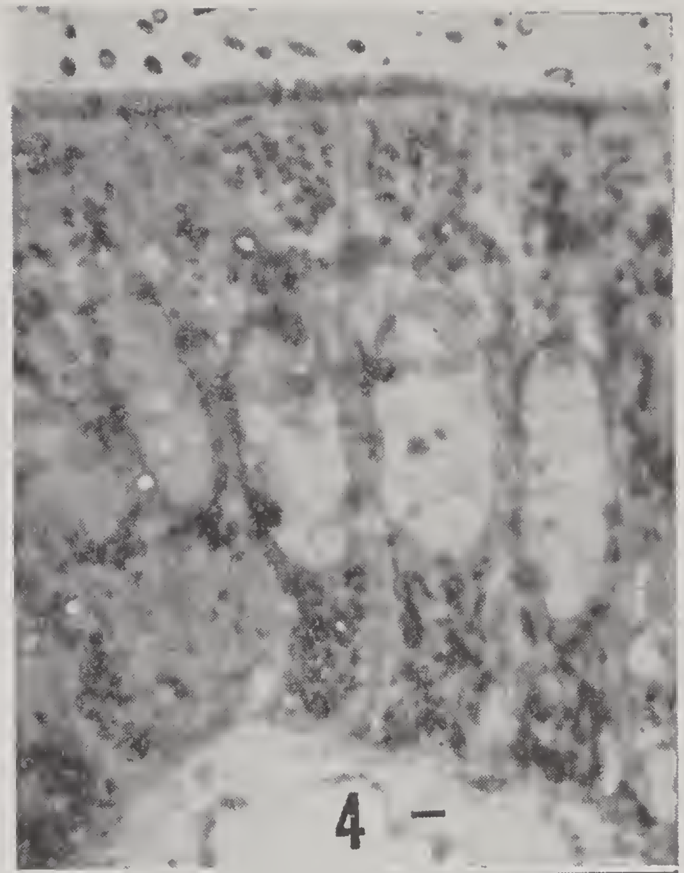


FIG. 3.—Part of a cell from the convoluted portion of a proximal tubule of a mouse kidney fixed as the cell in Figure 1, showing detail of the brush border. Approximately 11,000×.

There are several statements in the literature to the effect that removal of the embedding matrix before examination with the electron microscope, either following the double embedding method of Pease and Baker (1, 23) or the n-butyl methacrylate embedding method of Newman, Borysko and Swerdlow (18), results in the



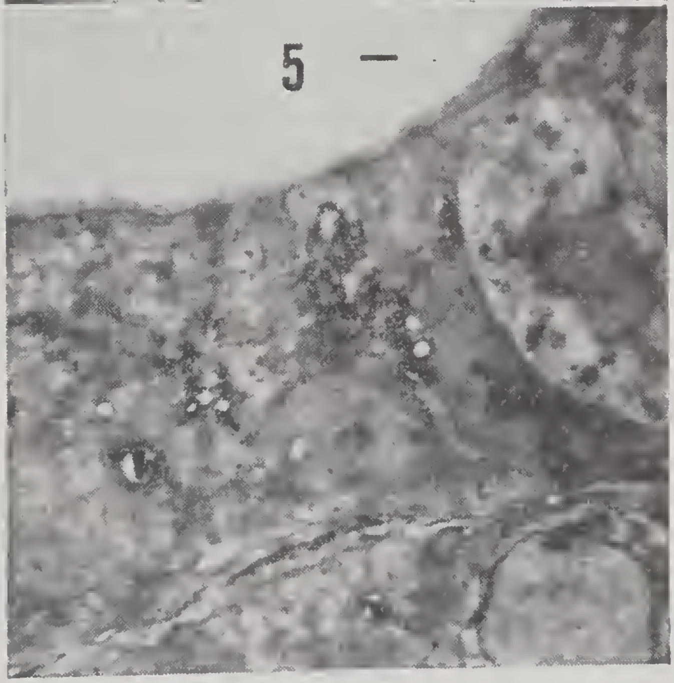


FIG. 4.—Several cells of the epithelium of the colon of a mouse perfused and fixed with Regaud's fluid followed by treatment with 2 per cent osmic acid at room temperature for 4 hr. Contrast is not as good as in Figures 1–3, but there is little evidence of distortion. Approximately 3,700×.

FIG. 5.—Cells from an intestinal gland of the duodenum of a mouse fixed by perfusion in Champy's fluid followed by treatment in 2 per cent osmic acid at 37 C for 3 days, showing the Golgi substance to consist of granular strands of osmiophilic material associated with empty vacuoles. Approximately 4,800×.

introduction of relatively gross artefacts. In our experience, fixatives containing osmic acid or fixation methods involving secondary treatment with osmic acid not only reduce such artefacts but also largely eliminate the damage which frequently results during the polymerization of n-butyl methacrylate.

Comment by Harrison Latta

The fixation procedure Dr. Dalton describes certainly avoids many artefacts apt to appear with other methods. Further evaluation of the effects of preparative procedures on cellular ultrastructure appears to require study of less complex protein systems, correlating the results of electron microscopy with those of other biophysical and chemical techniques. Indeed, most biologic problems which aim at interpreting cellular events in terms of molecular and macromolecular phenomena necessitate such a correlative approach.

Additional discussions on thin sectioning procedures include refinements on a conventional microtome (15), a triple embedding procedure (22), a double sectioning procedure (27) and a study of the kidney using a rocking microtome (19). In the last study, the observation (24) of ridges on the external surface of the glomerular capillary basement membrane was confirmed, and a honeycombed trellis on the interior of the membrane was associated with endothelial cells. Further experience with the glass knife (16) has shown that more consistent results are obtained if oil and dust are removed from the edge before use by dipping successively in an organic solvent, sulfuric acid-potassium dichromate cleaning solution, and distilled water and drying without wiping. Excellent electron micrographs have been obtained recently from several tissues prepared with a specially built microtome (21).

Various other important methods of preparing biologic specimens for electron microscopy are available or are in the process of development. Further references will be found in recent treatises on the practice of electron microscopy (8, 29) or in the bibliographies on electron microscopy (4, 17).

Comment by A. C. Corcoran

The fields of tissue electron microscopy and ultramicropathology are just opening. The technique described is one way in which kidney tissue may be prepared for study under the electron microscope. The enormous vascularity of the kidney undoubtedly facilitates the procedure, so that applications of this method in other organs may require adaptations of a technique which may, in its own right, illuminate some of the peculiarly dark and obscure corners of renal pathology.

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MEASUREMENT OF RENAL FUNCTION IN RATS

SMITH (14) HAS pointed out that, in view of the inherent technical difficulties, "it is surprising that five groups of investigators arrive at about the same figure for the filtration rate (0.6 to 0.76 cc. per minute per 100 grams BW)." Modes of collection of urine, of blood, of giving test substances, of establishing diuresis, all vary; variations in diet and differences in strain must also be reckoned with. The very number of techniques that have been described attests the desire of investigators to have methods of renal study which they can apply in small animals. Consequently, 3 methods are described here in the hope that some synthesis of procedures may resolve the issues which remain.

I. Measurement of Filtration Rate and Tm_{PAH}

G. M. C. MASSON *and* A. C. CORCORAN, *Cleveland Clinic Foundation*

Principle.—Estimation simultaneously of filtration rate from exogenous creatinine clearance ($C_{Cr\ exog}$) and of tubular secretory capacity in the rat: catheter collection of urine during osmotic diuresis, subcutaneous injection of test substances, estimation of plasma concentration by interpolation from 2 blood samples, light anesthesia before urine collection.

PROCEDURE

a) Apparatus.—(1) Rat holder with sliding sleeve adjustable to animals of different sizes. (2) Pipets (uncalibrated) for collecting blood; soft glass, 4 mm inside diameter. (3) Ureteral catheter, no. 4 F, about 15 mm long. (4) Warming box set in the range 40–45 C.

b) Solutions.—(1) Heparin in 1 per cent NaCl (2 mg/cc). (2) PAH-mannitol-creatinine: PAHNa 6 g, mannitol 10 g, creatinine 4 g—all per 100 cc of sterile water. (3) Optional.—Intracaine (Squibb), 2.5 per cent in 1 per cent NaCl.

c) Technique.—1. Female rats uniform as to strain and diet and weighing about 150 g are given an intraperitoneal injection of 0.3 cc of heparin solution and lightly anesthetized with ether.

2. Each rat then receives a subcutaneous injection of 1.7 cc of PAH-mannitol-creatinine/100 g body weight and the time is noted as 0. The bladder is catheterized and the rat is placed in a holder

adjusted to restrain movement. Intracaine can be injected into the bladder to relieve discomfort as anesthesia wears off.

3. At 40 min after 0 time, rat and holder are placed in the warmer for 3 min. The 1st blood (B1) is collected onto a heparinized watch glass from the snipped end of the tail and drawn up into a pipet, the dry end of which is sealed in a flame.

4. At 48 min after 0, the bladder is rinsed with 4 portions of saline (0.2–0.5 cc each); the last rinse is completed exactly at 50 min and all rinses are discarded.

5. From 50 to 58 min, urine is collected by drainage into a 5 cc graduated cylinder. Rinsing is then repeated as before, ending at 60 min, and the rinses are added to the urine sample; urine + rinse is then made up to 100 cc with water.

6. The rat is then warmed as before and blood (B2) collected as before, the collection ending at 65 min.

7. For serial determinations in a group, each succeeding rat should begin 0 time at intervals of about 25 min.

8. The pipets are then sealed at 1 end in a flame and the blood is centrifuged in them; they are then broken at the cell-plasma interface and 0.1 cc of clear plasma is secured for preparation of a $CdSO_4$-NaOH protein-free filtrate (1:100 dilution). PAH is determined in filtrate and diluted urine by the method of Goldring and Chasis and creatinine by an adaptation of the method of Folin and Wu. Mean plasma concentration during the clearance period is estimated by interpolation from a semilogarithmic plot of values of B1 and B2 against time.

d) Calculations.—These are made in the customary manner, using for Tm the value of 0.83 assigned to man.

Results.—$C_{Cr\ exog}$ averages 0.61 cc/min in Sprague-Dawley rats (2); a mean of 1.12 has been found in rats of the Wistar strain (12) and of 0.75 in the same strain on a creatine-creatinine–free diet. Mean Tm_{PAH} averages 0.29 mg/min in our Sprague-Dawleys, 0.47 in Wistars and 0.38 in Wistars on the creatine-creatinine–free diet (12). The values are stated in units/100 g body weight. Determinations repeated at intervals of weeks should not depart from the mean by more than 10 per cent (2).

Evaluation.—There are several advantages. Accuracy of urine collection is obtained by establishment of mannitol diuresis, catheterization and bladder rinsing; the brief test period permits accurate interpolation of plasma concentrations during the clearance period; artefacts due to anesthesia, trauma or blood loss are minimized by making urine collections under physiologic conditions.

Disadvantages are pointed out below. One which is not noted is the probable inaccuracy of the factor 0.83 for FW in the calculation of PAH; another is the tedious character of the experiment, which limits observations to 6 rats per morning or afternoon.

Comment by Richard W. Lippman

This method is similar to our "tail-cutting" procedure (9). The objection to it is that tail-cutting causes circulatory responses which extend to the kidney and persist longer than is allowed for in steps 3 and 4. We have found subcutaneous absorption of test substances irregular in the rat, so that even 1 hr after injection one cannot be sure of a drop in serum concentrations. We have also found that the high plasma concentrations necessary for determination of Tm_{PAH} depress inulin and creatinine clearances in the rat and for longer periods than in dog or man (10).

The validity of creatinine clearance as a measure of glomerular filtration in the rat has also been questioned. Martel *et al.* (12) found that C_{Cr}, determined as described here, fell slightly as plasma creatinine content increased, although the change is of doubtful significance. We had shown that C_{Cr} rises conspicuously as the transition is made from measurement of endogenous to exogenous creatinine (8), and Martel *et al.* (12) found exogenous C_{Cr} consistently greater than C_{In}. The ratio C_{Cr}/C_{In} Martel observed was consistent and of the same order as that we have found. Preliminary experiments (our laboratory and Martel) indicate that this ratio not only is stable under a variety of conditions but is also not altered by the giving of phlorizin. We have therefore continued to use it as a measure of glomerular filtration rate in the rat, aware that it may be found not to yield absolute values thereof.

Comment by S. E. Dicker

Disadvantages of the method are that it allows for the use of female rats only; more seriously, it involves anesthesia (5, 6), and measurements of renal function may be altered by mannitol diuresis.

Comment by Dr. Masson and Corcoran

Martel and his colleagues (12) modified our method by decreasing concentration of injected substances to about $^1/_4$ and by using only 1 end-point blood sample without, in their opinion and ours, substantially decreasing the accuracy of the method. The use of the 1 end-point blood, previously recommended by Braun-Menendez and Chiodi (1) for determinations of C_{In} and Tm_D, obviates Dr. Lippman's objection to preliminary tail-cutting. His objection to the use of PAH, since confirmed by Martel (12), stands, as do the disadvantages noted by Dr. Dicker.

II. "Undisturbed" Method for Clearance Determination

RICHARD W. LIPPMAN, *Cedars of Lebanon Hospital, Los Angeles*

Principle.—Measurement of exogenous C_{Cr} as an indicator of filtration rate: saline hydration, intravenous injection of test substances, interpolation of mean plasma concentrations from measurements in injected controls.

PROCEDURE (1, 11)

a) *Apparatus.*—(1) Metabolism cage and funnel. (2) Deflecting bulb and centrifuge tube (Fig. 1).

b) *Solutions.*—(1) 0.85 per cent NaCl. (2) 0.85 per cent NaCl containing 5 mg creatinine/1.7 ml. This solution is prepared in sufficient volume to serve for control and test groups in any single experiment.

c) *Technique.*—1. Rats of the same sex, uniform size and under stable dietary conditions are divided in 2 groups of 6. They are given 3 intraperitoneal injections of saline at 9:30 A.M., 4:30 P.M. and 9:30 A.M. the following day.

2. Five hr after the 3d intraperitoneal injection, the controls are given 1.7 ml of creatinine-saline by intravenous injection into the dorsal foot vein, using a tuberculin syringe and 25 gauge needle.

3. Two min after completion of the intravenous injection, each rat in this group is lightly anesthetized with ether, the abdomen opened and the rat killed by exsanguination from the abdominal aorta. The blood is collected for creatinine determination.

4. A similar intravenous injection from the same lot of creatinine-saline is given animals of the 2d group, which are then placed in cages for collection of urine. In either group, leakage from the injection site is controlled by application of a small rubber tourniquet held in place with a Dieffeback clamp for as long as need be, or the foot can be dipped in collodion.

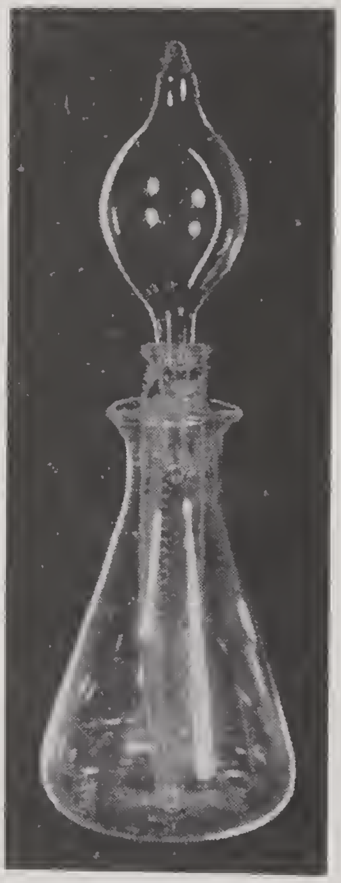

FIG. 1.

5. Urine collection is completed 15 min after the end of the intravenous injection by anesthetizing the test group rats with ether while they are *held in place* over the collection funnel. The abdomen is then opened and the rats are exsanguinated as in the control group. At the same time the bladder is examined and any remaining urine is evacuated by syringe and added to the urine previously collected.

6. Funnel and collection bulb are rinsed with small portions of saline and the rinse fluids added to the urine specimen.

d) *Calculations.*—Clearance is calculated from mean serum and measured urine creatinine concentrations. Mean serum concentration is calculated on the assumption that concentration decreases exponentially after intravenous injection of the test substance.

Let a = group mean serum creatinine concentration at 2 min; b = group mean serum creatinine concentration at 15 min; n = group mean serum creatinine concentration at the 15 min clearance period for any individual rat, and n_{15} = serum creatinine concentration of any individual rat at 15 min. Then, since the measured serum concentrations are 13 min apart, but the collection period lasts 15 min

$$\log n = \log n_{15} + \frac{7.5\,(\log a - \log b)}{13}$$

Thus, 6 individual clearances are obtained from each group of 12 rats.

Results.—Under these conditions a mean creatinine clearance of 1.36 ml/min/100 g body weight has been observed in rats of the Slonaker-Addis strain on creatine-creatinine–free diets. Sellers *et al.* (13) have obtained similar normal values by this method.

III. Determination of Inulin Clearance

A. C. CORCORAN

DICKER PROCEDURE

S. E. Dicker, University College, London, has summarized for us the method which he has devised.

Principle.—Determination of inulin clearance as a measure of glomerular filtration: establishment of water diuresis, subcutaneous injection of test substance, brief collection of spontaneously voided urine, and single (terminal) blood sampling.

a) *Apparatus.*—(1) Buret, 50 ml. (2) Soft rubber Jacques catheter, no. 41–3. (3) Glass funnel, 25 cm in diameter, fitted with feces separator which allows urine to collect into a finely graduated cylinder below.

b) *Solution.*—Inulin, 5 per cent in 0.85 per cent NaCl.

c) *Technique.*—Rats of at least 150 g body weight are used (3, 4). One is wrapped in a towel, a small gag placed in the mouth and the Jacques catheter, fitted at 1 end to the buret, pushed into the stomach, and through it is given 5 ml. of water/100 g body weight. This is followed immediately by subcutaneous injection of 1 ml/100 g body weight of inulin solution. The bladder is emptied 50 min later by prodding, by suprapubic pressure or both. The animal is then put in the funnel and urine collected for 5–7 min; since it is in full diuresis, it will void spontaneously. (Postmortem controls have always shown the bladder quite empty.) The rat is immediately anesthetized and blood sampled either from the carotid and jugular vessels or from the tip of the tail.

Evaluation.—Advantages are: (1) the procedure is done by 1 worker only; (2) only 1 blood sample is required (50 minutes after subcutaneous injection of inulin) the concentration in plasma does not vary significantly during the short period of urine collection (6, 7), and (3) anesthesia at the end of the collection period cannot affect renal function as measured.

Disadvantage is difficulty in administering water; Dicker writes: "after years of practice, I can manage 16 rats in less than 15 min."

FRIEDMAN PROCEDURE

S. J. Friedman, University of British Columbia, has summarized the procedure he uses in measurement of the plasma clearances of inulin and PAH (7).

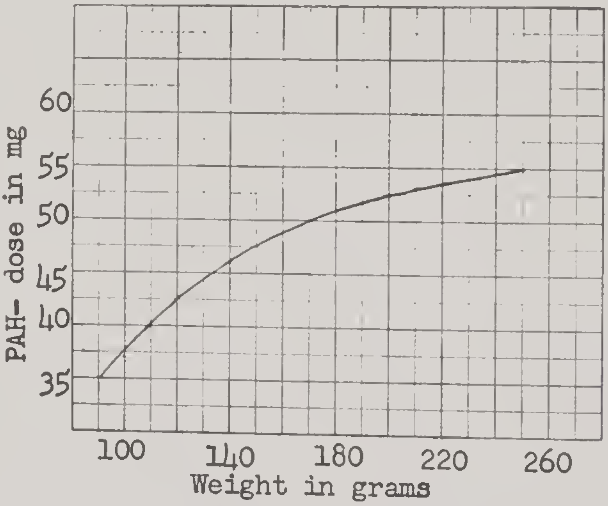

FIG. 1.—Dosage table for PAH administration.

Two solutions are prepared: (1) PAH, 12.5 mg/cc in 2 per cent sodium sulfate; (2) inulin, 2 per cent in 1 per cent NaCl.

PAH solution is injected subcutaneously in the lumbar region in an amount estimated from a dose table (Fig. 1) to yield plasma concentrations of 5–7 mg/100 cc. Immediately thereafter 3 cc of warmed inulin solution is given intraperitoneally. The rat is placed directly into a large funnel over a beaker and secured there by a lid. The bladder is emptied by suprapubic pressure 50 min later and the tail rinsed into funnel and beaker. Blood (0.75 cc) is then obtained by heart puncture, using a heparinized syringe and a

24 or 25 gauge needle; sampling should be completed by the 51st minute. The sample is prepared for analysis by centrifugation. The funnel is rinsed into the beaker and urine + washing made up with water to 100 ml. Any fecal admixture is removed by centrifugation of the diluted urine.

Friedman notes: "The procedure is not too accurate, but is satisfactory in large groups of animals. With a team of 2, we can do 16 clearances in the morning and as many in the afternoon." Values of C_{PAH} and C_{In} thus obtained average respectively 4.1 and 0.65 cc/min/100 g body weight.

PROCEDURE OF BRAUN-MENENDEZ AND CHIODI (*1*)

In this method, inulin and Diodrast clearances and Tm_D were determined after subcutaneous injection of solutions of test substances and initiation of water diuresis at 0 time by administration of water, equivalent to 5 per cent of body weight. The bladder is emptied by manual expression 1 hr later, the rat placed in a metabolism cage and the bladder again emptied manually if the rat has not completely voided spontaneously at about 30 min. The blood sample is then secured.

By this method C_D and C_{In} average, respectively, 2.66 and 0.60 cc/min/100 g body weight, and Tm_D (as iodine) 0.18 mg. Each function shows some correlation with urine flow.

General Comment

A. C. CORCORAN

The methods described here can perhaps best be evaluated in terms of advantages and disadvantages, and it is obvious that the accuracy of each is more dependent on pooling of data from groups than on extreme accuracy in individual animals. The obvious disadvantage of Lippman's method is the need for killing not only test, but also control, animals; serial observations are impossible and the waste would seem to some excessive. Technically, the inconvenience of intravenous injection, the possible effects of the anesthesia which may be required at the time of injection and some uncertainty as to the validity of calculation of clearance from estimated mean plasma concentration should be noted. During the few minutes after instantaneous injection of creatinine solution, there may occur volume effects on renal circulation and function, and, since the curve of creatinine in plasma is the sum of 2 exponential disappearances, respectively, by renal removal and diffusion in tissue fluids, the calculation of mean value may be in error, while also it does not allow for delay time in the urinary tract. Dicker's method seems by all odds the simplest in principle and as accurate as any in practice. Friedman's method may suffer from administration of PAH as a test substance, from the long periods of urine collection after injection and the choice of the end-point single blood sample as representative of this period. The method of Braun-Menendez and Chiodi has the advantage that urine is only collected after partial equilibration of blood levels and that the collection period is short and the blood drawn at the end of that time. However, again, the precision of an end-point blood sample for a 30 min clearance period can be questioned.

Thus, the points to be resolved in the estimation of renal function in rats seem to us to be as follows:

1. Can spontaneous voiding even during diuresis be relied on for complete collection of urine formed over short intervals?
2. If, as seems evident, some diuresis must be established, at what level and by what means (water, saline, sulfate or mannitol)?
3. What concentration of PAH will not depress glomerular filtration rate and what substance is to be used to measure true filtration rate in the rat?
4. If end-point blood must be used as the single sample, what can be done to stabilize blood concentration over the preceding collection period? How long should that period be? What allowances must be made for abnormalities of renal function?

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EXPERIMENTAL RENAL PATHOLOGY

Introduction

A. C. CORCORAN

The aspects of experimental renal pathology discussed here form a small segment of the whole topic. The selection is based on the premise, previously mentioned, that the rat has come to stay as a subject for the investigation of many problems in vascular and renal disease, although certainly not to the exclusion of other species. In particular aspects the vascular and renal, as well as social, responses of rats have perhaps more in common with those of human beings than do the reactions of other lower animals.

In particular, the measurement of arterial pressure in such an animal as the dog is a well established procedure, whereas this determination is a vexed issue in the rat. The tail plethysmographic method of Harrison *et al.* (2) has been repeatedly modified; in our hands, in much the same form as was described by Kempf and Page (1), it has been on the whole satisfactory. However, it is still a commonplace for investigators who willingly accept their own measurements made with this apparatus to reject as faulty the measurements made in good faith in other laboratories. The new methods described here have such definite advantages of end-point that it seems likely they will supplant the older procedures.

The establishment of hypertension in rats is a topic closely related to the measurement of arterial pressure and, as will be seen, seems somehow usually to involve the kidney.

Heymann's technique of eliciting nephrotoxic nephritis in rats interestingly concludes a chapter which, left to his pleasure, the editor might have filled in still other ways of causing, relieving and studying renal disease.

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I. Measurement of Arterial Pressure in Rats

MICROPHONIC MANOMETER FOR INDIRECT DETERMINATION OF SYSTOLIC BLOOD PRESSURE

MEYER FRIEDMAN *and* S. CHARLES FREED, *Mount Zion Hospital, San Francisco*

The method of obtaining the systolic blood pressure of the rat is similar in principle to that employed in the indirect measurement of systolic blood pressure in man, namely, the detection of sound at the exact time that the arterial pressure of the caudal artery exceeds the pressure of the occluding cuff. This method was found to be accurate, simple and rapid.

PROCEDURE

a) *Apparatus.*—This consists essentially of a carbon type microphone 3 cm in diameter to the diaphragm of which is attached a thin copper trough 1.5 cm long and 0.6 cm in diameter. The microphone is connected to a specially designed, low frequency (100 cps) sound amplifier (Fig. 1)* operated by direct current supplied by 3 dry cells. The amplifier affords a voltage gain of approximately 3,000. The energy changes obtained by the amplifier may be detected by ordinary earphones, loud-speaker or oscilloscope.

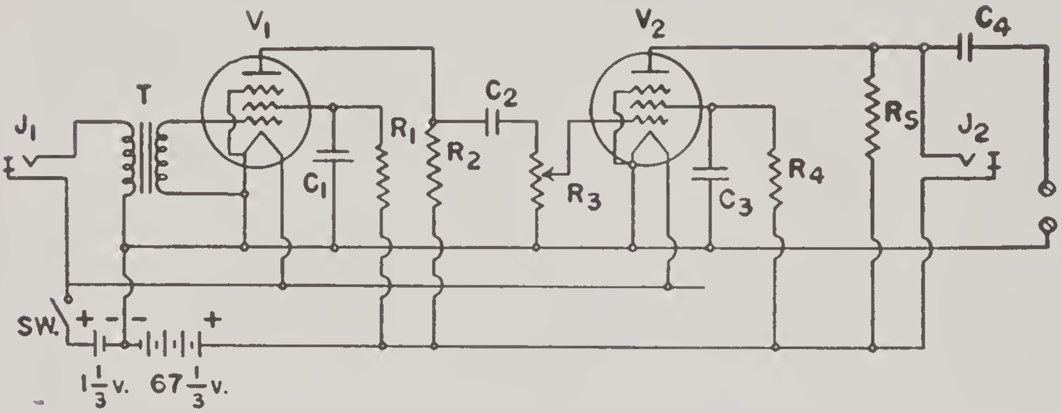

FIG. 1.—Electric circuit of amplifier. V_1, V_2, type IT4 vacuum tubes; *T*, Stancor type A-4705 microphone transformer; J_1, J_2, open circuit phone jacks; C_1, C_3, 1 mfd by-pass condensers; C_2, 0.1 mfd coupling condenser; C_4, 0.001 mica condenser; R_1, 1 megohm; R_2, 500,000 ohms; R_3, 1 megohm potentiometer; R_4, 100,000 ohms; R_5, 100,000 ohms.

b) *Technique.*—To take blood pressure readings, a rat is warmed 5–10 min in a wood box, the temperature of which is maintained at approximately 37 C. The tail of the rat is led through a 10 mm

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* Further details of construction of this apparatus and the electrical circuit will be furnished by the authors on request. The microphonic manometer itself may be obtained from Charles Calvert of the Pacific Industrial Electronics Company, 378—28th St., San Francisco, Calif. The cost is approximately $200.

pressure cuff and the segment of the tail immediately distal to the cuff is placed in the trough and fixed by a strip of adhesive tape. The pressure of the cuff is raised with a rubber bulb to a point above the expected blood pressure, then slowly released. At the point at which the cuff pressure becomes less than the caudal arterial pressure, one hears *immediately* in the earphones a rhythmic succession of sounds reflecting the transmitted pulsatile variations of the caudal artery. If the amplifier is connected to an oscilloscope, one sees at this same critical point an intense and abrupt change in contour and frequency from the preceding waves. It should be stressed that the regularity and actual rate of these sounds do not allow their confusion with any possible extraneous movements of either the body or the tail of the rat. As the cuff is deflated further, the sounds attain their greatest intensity at the expected diastolic level, although this increase in intensity is too gradual to allow precise determination of diastolic pressure. The sounds continue even when the cuff is completely deflated.

Preliminary venous drainage of the tail is not necessary as the method depends not on gradual increase in tail volume but on the almost instantaneously transmitted pulsations which occur when the cuff pressure is overcome.

Comment by W. Glen Moss

My impression is that the combined "audiovisual" procedure is the best indirect method available for determining blood pressure in the rat. Difficulties may be encountered from respiratory and other movements which are transmitted through the rat's tail and the framework of the holder and chamber to the microphone. We have overcome most of these by seating the microphone over rubber and applying a rubber-lined clamp laterally to the tail just before it enters the cuff. In some rats with experimental renal hypertension, we have heard pulsations when the cuff pressure was well above systolic blood pressure. This seems to be due to a partial shielding of the "constricted" caudal artery by the small processes on the caudal vertebrae; however, this is only a theory. If the tails of such animals are warmed by washing in water at 45–50 C and the measurement repeated, a satisfactory reading can usually be made, presumably because the caudal artery has vasodilated. The procedure is readily taught, and by using 3 rat holders in rotation, a rate of 20–40 measurements/hr can be achieved.

In rats with experimental hypertension in which sounds can be heard at all cuff pressures, the same difficulty is found in attempting to determine systolic pressure by the plethysmographic technique. Under anesthesia, the blood pressure recorded by direct artery puncture is within the range of the manometer used in the cuff method. It is because of this that I believe the caudal artery in such rats is "protected" from the pressure applied through the cuff. Therefore in such animals it would seem that the only accurate means of measuring systolic pressure is by cannulation or artery puncture. I have not investigated this difficulty in measuring the blood pressure of hypertensive rats any more thoroughly than stated here.

RECORDING OF INDIRECT BLOOD PRESSURE IN THE UNANESTHETIZED RAT

FREDERICK OLMSTED, *Cleveland Clinic Foundation*

Graphic registration of foot pulse in the rat, with simultaneous record of air pressure in an occluding cuff proximal to the point of pulse pick-up, offers a method of blood pressure determination in this animal free from subjective error, with the advantage of a permanent recording for each analysis. The method has been used, in addition to normal and hypertensive white rats, in Kangaroo rats, parabiotic rats and in rabbits; with minor modification, it seems applicable to most animals. In the rat, foot arteries must be at least normally dilated to insure a clear pulse recording and adequate compression of the pulse. Measurements are therefore made at an ambient temperature of 30 C. To avoid the pressor effect of abrupt heating (4), the animals are placed in this environment several hours before measurements are made.

PROCEDURE

a) *Apparatus.*—The rat holder, foot platform and electrical pick-up are shown in Figure 1. The center section of the holder is brass tubing; this varies from $1\frac{1}{2}$ to $2\frac{1}{2}$ in. in diameter to accommodate animals of different sizes. The front section is a heavy bronze mesh cone soldered to a brass cylinder which slides snugly in the center section. The rear part is constructed of inner tube rubber, with the leg hole so positioned that the rat's hip is locked in place by gentle squeezing of the rubber sides at the location of the clip. The unbonded strain gauge* and foot platform are mounted at an angle of 45 degrees on a heavy metal slab that can be maneuvered horizontally to accommodate the foot. The oilcloth strap is $^3/_8$ in. wide and acts both as foot holder and pulse pick-up.

The electrical circuit consists of slightly modified standard equipment (Fig. 2). With minor alterations, the stage following the Grass preamplifier consists of the Brush a-c amplifier for use with the Brush Penmotor. The arrangement gives a 3 cm pen deflection for a 50 μv^2 wave applied at the output terminals of the strain gauge. However, any amplifier with a voltage gain of 6×10^6 with response attenuated 95 per cent below 5 and above 35 cps, and impervious to surge currents is satisfactory if the output impedance matches that of the ink oscillograph used. It is advisable to locate the sensing element physically so that microphonic interference is avoided.

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* Model #G1-16-250, made by Statham Co., 12401 W. Olympic Blvd., Los Angeles.

The recording unit is a Brush Penmotor mounted at one side of a 2-channel chassis. The paper width (double channel size) is 8.5 cm to allow easy determination of cuff pressure. The receptor selsyn fixed in the unoccupied Penmotor space[†] (GE #2JIFI, 110 v, 400 cps, run on 25 v, 60 cps) actuates the cuff pressure pen normal to the direction of paper travel along a guide wire by a continuous thread belt driven by a drum on the motor shaft.

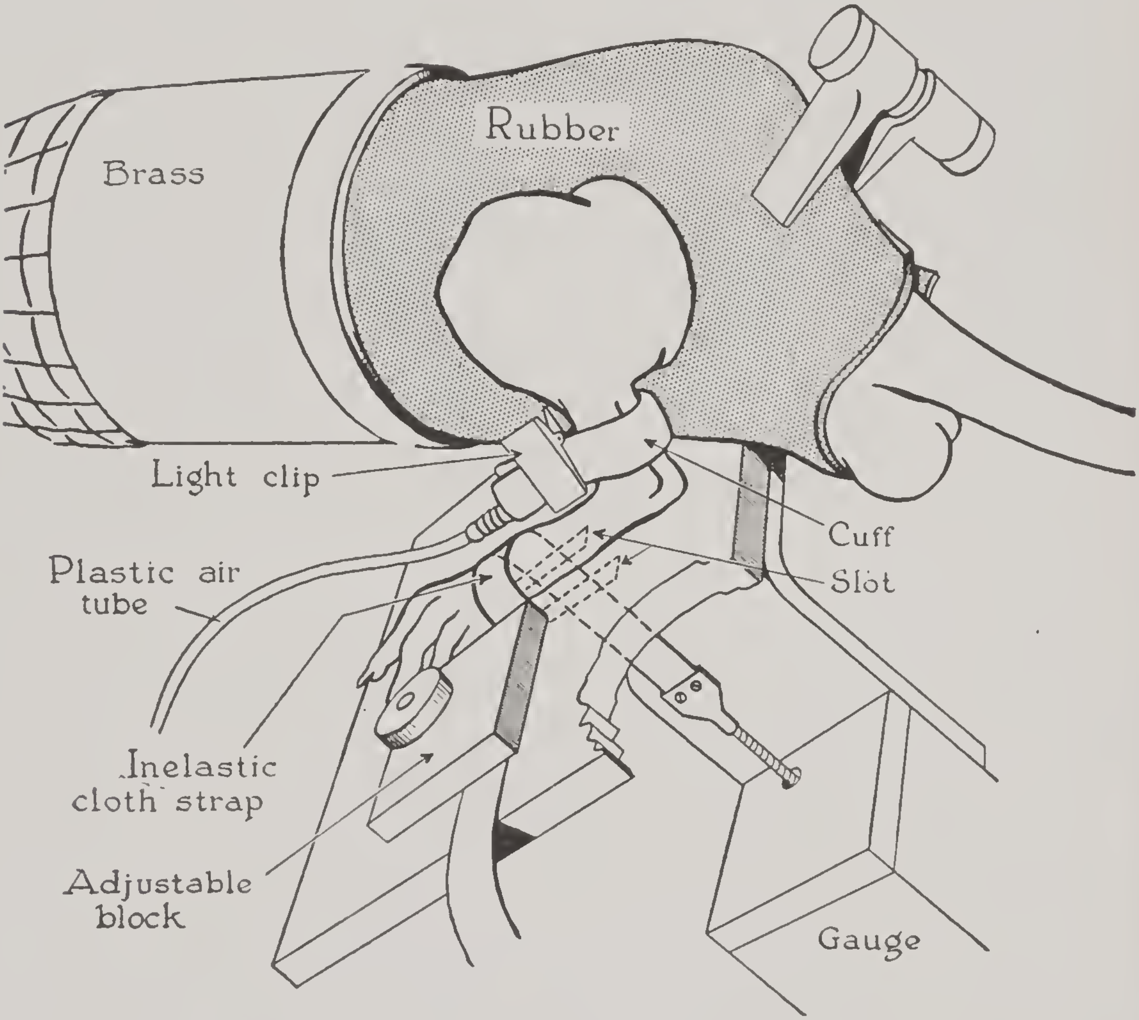


FIG. 1.—Animal holder and strain gauge mounting with animal in place (4).

The driver selsyn is activated by a 4 in. sylphon bellows in the pressure line opposed by a helical spring. A simpler but less convenient arrangement would be to drive the pen directly from an air bellows mounted on an oscillograph chassis. The air pressure pen is calibrated stepwise with a mercury manometer, its range being from zero to 250 mm Hg.

[†] Metro Industries, 29-28 41st Ave., Long Island City 1, N. Y.

The cuff described by Kersten and co-workers (3) and modified by a manufacturer† is satisfactory for use with the apparatus. A simpler and more easily applied, but probably less accurate,

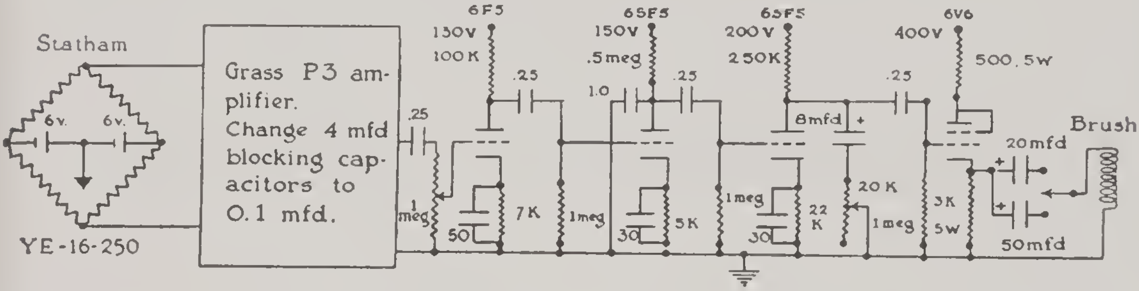

Statham
6v.
6v.
YE-16-250
Grass P3 amplifier.
Change 4 mfd blocking capacitors to 0.1 mfd.
.25
1 meg
6F5
150V
100K
.25
50
7K
1meg
6SF5
150V
.5meg
1.0
.25
30
5K
6SF5
200V
250K
8mfd
+
1meg
30
22 K
20K
1meg
.25
6V6
400V
500. 5W
3K
5W
20mfd
+
+
50mfd
Brush

Fig. 2.—Electrical circuit (4) as described in text. The band pass type of circuit was found impractical.

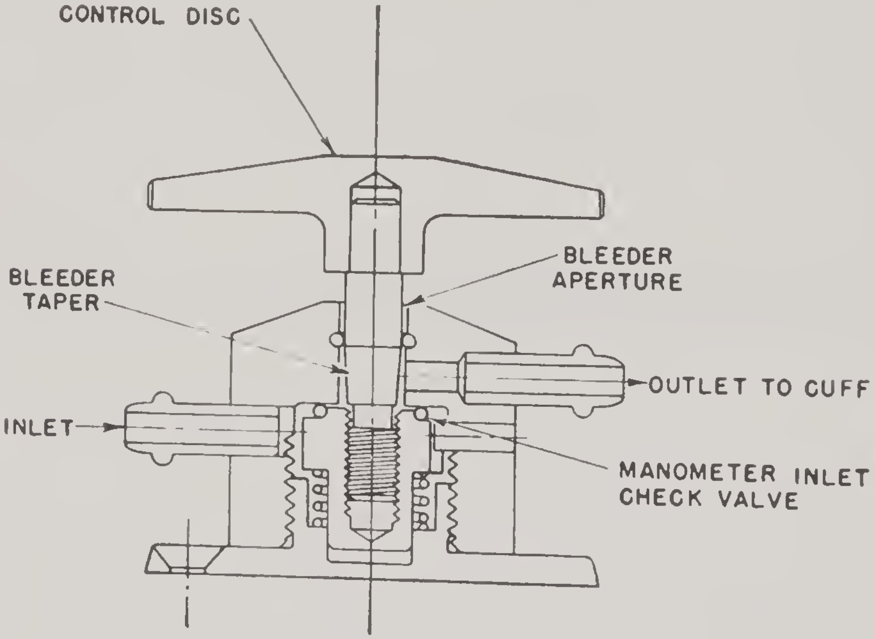
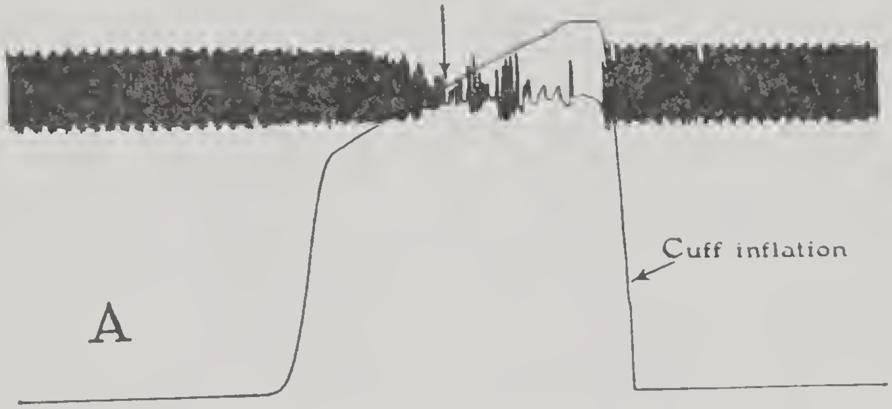

CONTROL DISC
BLEEDER TAPER
BLEEDER APERTURE
OUTLET TO CUFF
INLET
MANOMETER INLET CHECK VALVE

Fig. 3.—Valve for rapid inflation and controlled slow deflation of occluding cuff (4).

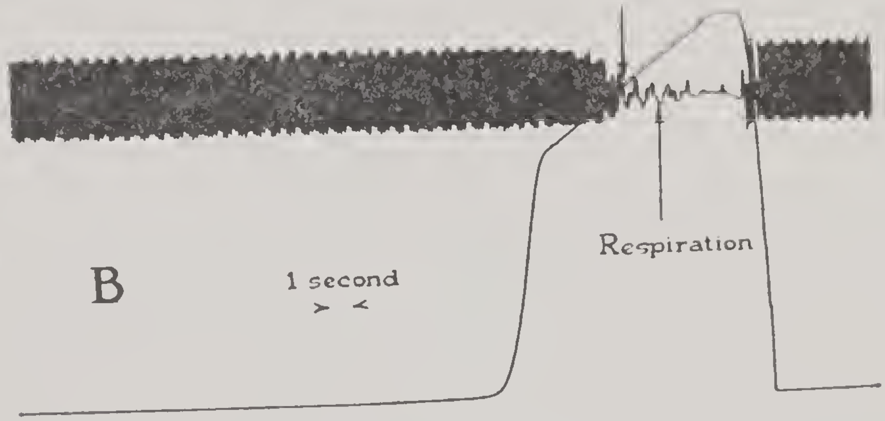
cuff may be constructed of $^1/_4$ in. Penrose tubing (4). Its effective length is 1 in. It is backed by a $^1/_2$ in. width of heavy dental dam, and a small-bore (18 gauge) polyvinyl tube supplies air pressure. To apply the cuff, a single loop is made around the rat's leg and fastened with a miniature clip. However, unless consistent tension is maintained from animal to animal and from time to time in fitting this cuff, blood pressure readings may be affected.

Air pressure used in this method is supplied by laboratory air lines reduced by a commercial valve‡ to 250 mm. Hg (about 5 lb/sq in.). Air is admitted to the cuff and recording bellows by a special valve§ (Fig. 3) whose action is such that pushing the control disk down inflates the cuff rapidly, and releasing it starts a gradual leak from the system, finely regulatable by rotation of the control disk.

b) *Technique.*—Although the foot pulse method seems at first to involve more manipulation of the animal than some other methods or their variations now in use (1, 2, 5, 6), with practice a half-score of readings may be obtained at the rate of 10 min/animal, with the advantage that results recorded during periods of fluctuation of arterial pressure (4) may be discarded.



RESTLESS ANIMAL



QUIET ANIMAL

FIG. 4.—Sphygmograms illustrating systolic points at downward pointing arrows (4).

The rat is placed in a proper sized holder (one that seems a bit too small for the rat to be tested) and the front cone pushed in until the animal is firmly but not uncomfortably compressed. The left leg is brought through the side hole, and the hip locked outside, as in Figure 1. The rear part is then refastened to secure this locking position. The holder is attached to a large buret clamp on a fixed vertical rod and the gauge platform moved to support the foot without altering its natural position. The pick-up strap is next placed over the foot at about the end of the metatarsal bones. When the adjustable block is clamped, the strap should not touch the sides of the slot in the platform. Tension should produce a snug fit around the foot, but not enough to distort its shape, agitate the rat or occlude venous return.

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‡ Model #40, Harris Calorific Company, Cleveland.

§ Micro Metric Instruments Co., 2891 E. 79th St., Cleveland 4.

The recorder is turned on for inspection of the pulse tracing. If this is satisfactory, the machine is halted while the cuff is applied. Readjustment of the strap may then be necessary. To obtain a sphygmogram, record 1 or 2 in. of clear pulse beat and depress the valve disk until the cuff pressure rises to its limit or until the pulse tracing disappears, leaving only artefacts of respiration and animal movement. Then rotate the valve control disk counterclockwise until the cuff pressure starts an appropriate rate of fall.

The relative systolic point is taken at the 1st reappearance of the pulse wave, which forms the apex of a cone of rhythmic pen fluctuations. Readings may be recorded as rapidly as this cycle of operations can be repeated, with the cuff pressure allowed to fall to zero between measurements (Fig. 4).

NOTE.—This section was reviewed by Simon Rodbard.

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II. Establishment of Hypertension in Rats

Experimental hypertension of varying severity and seemingly of distinct mechanisms can be established in rats by (1) initiation of a persistent change in renal circulation, as by compression of the renal artery or of the renal parenchyma (renal hypertension), (2) administration of substances possessing hormonal activity (hormonal hypertension), and (3) provision of excess sodium (salt hypertension). In each instance (described in the following articles), the ultimate mechanism of hypertension is obscure; however, each of these forms of hypertension has some renal connotation.

HYPERTENSION CAUSED BY PERINEPHRITIS

G. M. C. MASSON *and* A. C. CORCORAN, *Cleveland Clinic Foundation*

Principle.—Placing the kidney in a rigid shell results in a change in renal circulation of the blood and tissue fluid which is apparently distinct from ischemia as such and which causes persistent elevation of arterial pressure (2).

The foreign body reaction of the rat to perirenal application of silk or cellophane is much less severe than in the dog, and the fibrocollagenous hull that forms is consequently thinner. The tension with which the kidney is compressed by the wrapping at the time of its application is therefore a determinant of the success of the procedure. This precise tension must be learned by experience.

PROCEDURE

1. Rats weighing 70–100 g are selected because in the period of body growth, growth of the kidney tends to increase the compression exerted at operation. Males are preferred, because of the ovarian fat pads around the caudal pole of the kidneys of females.
2. A jar is prepared for administration of ether anesthesia.
3. Surgical scissors, large forceps, trachoma forceps, a probe, suture needles and cotton thread are made ready.
4. A set of rectangular pieces of closely woven silk of sizes varying around 35 × 25 mm with running stitches at the edge is made up. From each silk piece the 2 ends of the running purse-string stitch are left free for tightening at the time of application.
5. The rat is anesthetized and placed on its side. The lumbar area is shaved and the skin cleansed with alcohol.
6. Skin and muscle wall are cut open parallel and about 1 cm lateral to the vertebral column.
7. The dissecting forceps are used as retractors and the trachoma forceps inserted and used in exteriorizing the kidney. The kidney is

freed of its surrounding fat by the probe without damaging its capsule. A silk square which will approximately fit the kidney is then applied to it, and the ends of the running stitch around the edge are pulled and tied. The wrapping should cover the whole kidney. It should not extend over the renal pedicle, nor should the pedicle be involved in the tie. The kidney is then placed back in the abdominal cavity and the muscle and skin wounds are closed with a single suture.

Results.—The severity and incidence of the hypertension which follows perirenal application of silk vary. If only 1 kidney is wrapped and the other is intact, hypertension results in about 20 per cent of the animals, of which only occasional ones will show arterial pressures greater than 200 mm Hg. If the contralateral kidney is removed at the time of kidney wrapping, initial mortality from uremia will be large, but animals that survive the 1st postoperative week usually have severe hypertension which, in a few weeks, enters a malignant phase and causes death. If both kidneys are wrapped, hypertension of long duration and moderate severity appears in about 80 per cent, so this is the most generally satisfactory method. Periodic checks of pressure should be made during the first 3 weeks after operation in the last class of animals. Hypertension should have been sustained for 3–4 weeks before it can be considered stable.

Other methods.—Abrams and Sobin have established renal parenchymal compression with latex capsules (1) and Rau by the application of a plastic in a solvent (3). We have no experience with either method; on the face of it, the method here described seems simpler and, with practice, as effective as any.

Note.—This section was reviewed by Simon Rodbard.

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SMALL ADJUSTABLE RENAL ARTERY CLAMP FOR PRODUCTION OF CHRONIC SYSTEMIC HYPERTENSION

DONALD E. GREGG, *Army Medical Service Graduate School*

A small, adjustable silver clamp modeled after the larger Goldblatt clamp (1) has been designed and applied to the renal arteries of the rat to induce hypertension (Fig. 1). The clamp (4) is 1.7 mm high, 1.65 mm long and 1.5 mm wide.

PROCEDURE

For application of the clamp, the renal arteries are exposed with aseptic precautions through lumbar incisions and under pentobarbital sodium anesthesia (4 mg/100 g intraperitoneally). A clamp is generally applied at 1 operation to each renal artery after it has been cleared for a distance of 3–5 mm, about 1 mm from the aorta. The removable plate of the clamp is taken out and held with a pointed hemostatic forceps. With a curved, blunt-pointed iris forceps modified to hold the clamp, the inverted clamp is slipped under and up around the renal artery. The removable plate is seated and aligned. The clamp is then rotated through 180° to bring the screw into view. The screw is then turned down to just give complete occlusion and then backed off about $1\frac{1}{2}$ turns with a small jeweler's screw driver.

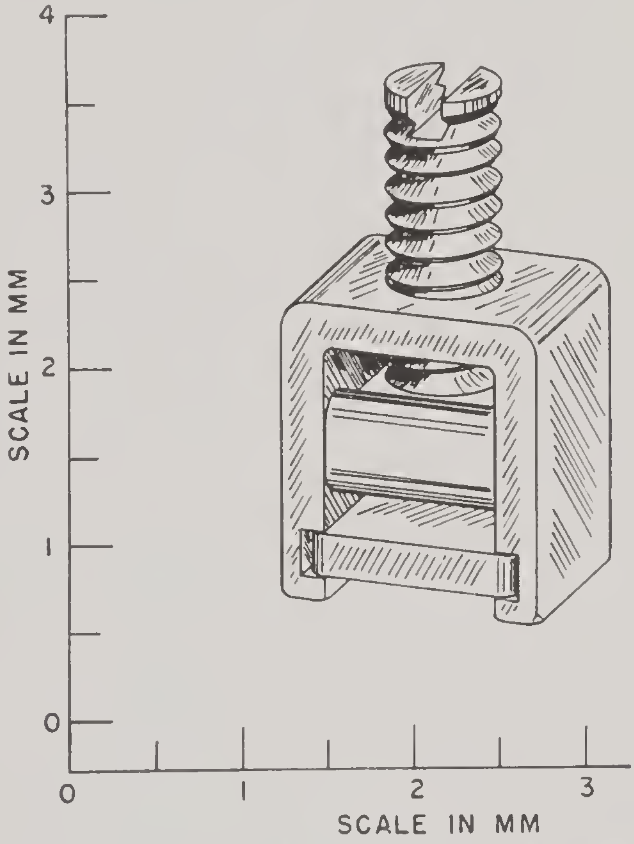

Fig. 1.—Dimetric drawing to scale of the adjustable renal clamp.

Results.—After application of the clamps blood pressure values, as determined in the hindleg of the unanesthetized rat by a modification (3) of the method of Kersten *et al.* (2), increased from control levels of 100–125 mm Hg to at least 160 mm Hg within a period varying from as short as 8 days to as long as 21 days postoperatively (3). The pressures remained elevated (160 up to a maximum of 220 mm Hg) for months. With careful technique $^3/_4$ or more of the rats in different series developed hypertension of this degree and duration. As indicated macroscopically and microscopically, the kidneys of the rats were generally normal, comparing favorably with those of unoperated controls.

Since this method gives a high percentage of hypertensive rats, it is particularly useful for study of small groups of rats. Although the clamps can be used repeatedly on different rats, expense of construction precludes their use on large groups of animals.

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HORMONAL HYPERTENSION

G. M. C. MASSON *and* A. C. CORCORAN, *Cleveland Clinic Foundation*

Among substances having some of the actions of hormones, 11-desoxycorticosterone (DC) or its acetate (DCA), 17-hydroxydesoxycorticosterone (compound S), diethylstilbestrol, thyroxin, crude anterior pituitary powder (APP, lyophilized anterior pituitary or LAP) and growth (somatotrophic) hormone have been reported to induce hypertension which, in certain instances, is associated with diffuse vascular disease and nephrosclerosis.

Reports on the hypertensive properties of cortisone and compound F in the rat are relatively indefinite, largely because the experiments are complicated by cachexia and infection.

Our own experience is limited to the use of DCA (2, 5), compound S (3), LAP (2), growth hormone (4), cortisone and compound F (1). In our hands, compound S does not induce hypertension; the hypertension caused by LAP and by growth hormone is variable both in degree and in incidence. Since the hypertension elicited by administration of DCA is relatively predictable in its course and reproducible in its occurrence, it is selected for description here.

Principle.—The hypertension which results from administration of DCA seems to result in some way from the sodium-retaining effects of this steroid.

PROCEDURE

1. Male and female rats weighing 60–80 g are selected and the left kidney is removed, since uninephrectomy sensitizes to the action of DCA.

2. The diet given is composed of commercial pellets; a solution of 1 per cent NaCl is substituted for drinking water.

3. The effectiveness of DCA depends on slow and constant absorption of the steroid. This is most economically secured by subcutaneous implants or by repeated injection of the steroid acetate as a water suspension. The more convenient method is introduction of 2 pellets of 35 mg each of DCA powder prepared as flat disks 6 mm in diameter. These are prepared in a suitable die and press. The press consists of a hydraulic auto jack firmly attached to a heavy base and supplied with a gauge connected with the oil reservoir. Depending on the consistency of the DCA powder supplied, the necessary pressure varies between 1,000 and 2,000 lb/sq in.

4. The pellets are implanted at the time of uninephrectomy; blunt forceps are pushed in the subcutaneous tissue from the skin incision forward to each scapular region and a pellet is then inserted at the end of each of these tracks.

When water suspensions are preferred, the steroid is dissolved in a minimal volume of hot alcohol and precipitated out by addition of excess 1 per cent NaCl in water. Crystals which may form can be broken down by shaking with glass beads; a drop of detergent solution is added to maintain the suspension. The daily dose of 2.5 mg DCA is then given subcutaneously in 2 doses.

Results.—Arterial pressure will be found to be increased by about 30 mm Hg in 10–12 days, at which also the animals manifest definite polyuria. Hypertension, with pressures greater than 150 mm Hg, will be present in all the animals by the 20th day. In some animals the disease pursues an acute course; in others the onset of manifestations of severe cardiovascular disease is delayed 8–12 weeks. These manifestations include arterial pressure greater than 200 mm Hg, tics and convulsive phenomena and evidence of mesenteric arteritis and of advanced nephrosclerosis.

NOTE.—This section was reviewed by Simon Rodbard.

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SALINE HYPERTENSION

G. M. C. MASSON *and* A. C. CORCORAN, *Cleveland Clinic Foundation*

Administration of excess sodium in the form of sodium chloride induces hypertension in rats (1, 2). We consider DCA hypertension a special and more severe form of salt hypertension, aggravated by the sodium-retaining properties of the steroid. However, since the procedure and the results differ significantly, the NaCl method is described here.

Principle.—Sodium excess induces hypertension in rats sensitized by uninephrectomy.

PROCEDURE

1. Rats weighing 200–300 g are selected and subjected to left uninephrectomy. Large animals such as these object less strongly to substitution of hypertonic NaCl for drinking water and have less diarrhea and loss of weight during its administration than smaller animals.
2. It is sometimes advantageous to start by substitution of 1 per cent NaCl for water; however, it is possible to proceed without excessive mortality directly by administration of 1.75 per cent NaCl and, after 3 or 4 weeks, substitution for this of a 2 per cent solution.

Results.—Mean blood pressures of about 160 mm Hg (range 140–200) will be found in the survivors after 2 months on a hypertonic régime, during at least 4 weeks of which 2 per cent NaCl has been given. This hypertension persists as long as treatment with hypertonic saline is continued. In our experience, it differs notably from DCA hypertension in that mesenteric vascular lesions do not occur and nephrosclerotic activity is minimal or absent. Whether or not this speaks for a pathogenic difference rather than a mere difference in severity is unknown.

NOTE.—This section was reviewed by Simon Rodbard.

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III. Nephrotic Syndrome Induced by Injection of Anti-Kidney Serum

WALTER HEYMANN, *Western Reserve University*

For the experimental production of renal disease in laboratory animals heteronephrotoxic antibodies were used for the first time by Lindemann (9) in 1900. Various workers used the idea subsequently. A thorough study of this type of renal disorder was made by Masugi (10) in 1923 in rabbits and by Smadel, Swift and Farr (13) 8 years later in rats. It was felt, however, that the value of these studies was limited because the experimentally produced disease did not seem to correspond clearly to any of the various forms of nephritis observed in man. In 1948, and in more extensive studies in 1951, Heymann and Lund (2, 3) showed that the disease produced in rats by the injection of anti-rat-kidney sera obtained from rabbits simulates the nephrotic syndrome—nephrosis with and without nephritis (1)—as it is seen in infants, children and, less frequently, in adults.

The disorder is characterized in rats given injections of rabbit serum by immediate onset of massive proteinuria without gross hematuria, with edema, ascites, hypoproteinemia and hyperlipemia. An initial azotemia subsides in the first 2–4 weeks. Spontaneous cures do occur, but more frequently proteinuria continues 1–1$^1/_2$ years without further injections. Hypertension, azotemia and creatinine retention may develop during the later stages of the disease and the glomeruli then show thickened basement membranes, adhesions and possibly fibrosis and hyalinization. Many of the animals, however, remain free from clinical evidence of progressive glomerular involvement and histologically the glomeruli are of normal appearance. One and the same experimental procedure thus may produce nephrosis in its so-called "pure" or "mixed" form.

Heymann showed that the antigenic property of renal tissue is localized in the renal cortex (5). Solomon and colleagues (14) found soon thereafter that suspensions of glomeruli were antigenic, whereas suspensions of tubular proteins were inactive. Krakower and Greenspon (8) have separated the basement membrane from the glomerular tufts and reported suspensions of basement membranes to be 50 times as antigenic.

PROCEDURE

a) Preparation of antisera.—Antisera against rat kidneys are prepared by injecting suspensions of perfused rat kidneys into rabbits. For perfusing the kidneys, 0.9 per cent NaCl solution is run into the thoracic aorta at a hydrostatic pressure of 1 m until the kidneys are grossly free from blood. When the perfusion fails, the animals are discarded. The kidneys are then removed, weighed and 0.9 per cent NaCl solution is added to make a 50 per cent suspension. A fine suspension is obtained by placing the material in a Waring Blendor for 5 min. After toluene and penicillin, 1,000 units/cc, are added the suspension is placed in an incubator at 37 C for 24 hr. The material is cultured and preparations giving any bacterial growth are not used. The suspensions are stored frozen. Rabbits weighing about 2,500 g are given intraperitoneal injections of 10 cc of a 10 per cent, 15 per cent and 20 per cent suspension every other day for the 1st week. Twenty per cent suspensions are used for the 2d and 3d weeks. Six days after the last injection 20–30 cc of blood is obtained by cardiac puncture. This treatment may be repeated after 2 weeks' rest 3–5 times in the same rabbit. The procedure almost regularly yields highly nephrotoxic sera.

TABLE 1.—Amount of Nephrotoxic Serum Injected into Rats, Gauged According to Kidney Weight

Wt. of Rat, g	Wt. of Kidneys, g	Amt of Serum, cc (R)
40	0.45	0.63
50	0.55	0.77
60	0.65	0.9
70	0.75	1.0
100	1.0	1.4
150	1.30	1.8
200	1.55	2.1
250	1.75	2.4
300	1.95	2.7
350	2.15	3.0
400	2.3	3.2

The rabbit serum is heated at 56 C for $^{1}/_{2}$ hr to diminish its primary toxicity. It is then stored frozen until used. Biologic assay is thus far the only means of determining the amount of nephrotoxin in an antiserum.

b) Analysis in rats.—It has been shown that after intravenous injection, renal antibodies are rapidly and selectively absorbed in the kidneys (11). It thus is advisable to gauge the amount of nephrotic serum to be injected into a rat according to kidney weight rather than body weight. A series of kidney weights of healthy rats weighing 30–50 g is obtained from Table 1 and plotted. The weight of both kidneys thus can be estimated in any rat to given injections, and 1.4 cc of serum (R-dose), fractions or multiples thereof, depending on the nephrotoxic activity of the serum, per 1 g renal tissue is injected. One third of the dose is given by intravenous injection on each of 3 consecutive days.

Extensive experiences have been obtained with rats of the Long-

Evans and Whelan strain. Their susceptibility to the disease does not differ. Rats of the Whelan strain have, however, a greater tendency to a progression of the glomerular lesion to fibrosis and atrophy (6). The younger rats have a greater tendency to anasarca. They may be given injections at the age of 4 weeks, weight approximately 50–60 g, 1 week after having been weaned. Quantitative determination of the 24 hr protein excretion is recommended. The physiologic proteinuria of the rat that varies with the strain of rats used and a dependence of the excreted urinary proteins on the urine volume make the quantitative estimation of qualitative tests for urinary proteins unreliable. For quantitative estimation of urinary protein excretion the method of Shevky and Stafford (12) is practical and reliable. For collection of urine the rats are placed in metabolism cages. The receptacle is to be covered with a rubber ring to prevent evaporation. For preservation of formed elements it should contain 2 drops of a 6 per cent Formalin solution. Methods for taking the blood pressure of rats are described elsewhere. We have been using the procedure of Kersten, *et al.* (7). Age-conditioned differences in the systolic blood pressure of the rat have to be taken into account and normal values, obtained with that method, have been established for rats weighing 50–400 g (4). For rats weighing 50–100 g, not more than 90 mm Hg should be considered normal; for rats weighing 100–350 g, 120 mm Hg, and for rats weighing over 350 g, 140 mm Hg.

Comment by Abbie I. Knowlton and Beatrice C. Seegal

In our experience the course of the disease and type of renal lesion produced by rabbit anti-rat kidney serum parallels closely that reported by Dr. Heymann, although early marked fluid retention has been observed in relatively few of our rats so treated. The technique employed in producing and administering the serum in our laboratory is essentially similar to Dr. Heymann's, but a few minor differences should be mentioned as these may possibly account for differences in our results.

1. After mincing the rat kidneys in a Waring Blendor, we store the material immediately in the frozen state, omitting the 24 hr incubation at 37 C which Dr. Heymann has employed. It is possible that this period of incubation produces certain changes in the antigenic properties of the kidney.
2. In our laboratory the immunized rabbits are not bled until after 3–4 courses of injections, and subsequently are bled, following provocative immunization, for periods up to a year or more. Dr. Heymann, on the other hand, has bled rabbits after each course of injections.
3. We have pooled the sera obtained from several individual rabbits to obtain large volumes of a uniform character. This may be of real importance in explaining the lower incidence of edema seen, since a single edema-producing serum may well have been "diluted" by such pooling. Dr. Heymann reports that he has tested each serum separately.

4. We have used, on the whole, older animals than Dr. Heymann and relatively smaller amounts of anti-kidney serum.

It should be mentioned that we have seen the acute edema in Sprague-Dawley as well as the Long-Evans strain of rats. We have determined blood pressure during the course of the disease. In agreement with Dr. Heymann, we have not seen hypertension during the early phase, even in severely edematous animals, but have observed it in the later phases (3–12 months). In severely edematous rats we have obtained markedly elevated nonprotein nitrogen values within a few days of serum administration.

Comment by Dr. Heymann

Although the factors indicated in comments (2) and (3) may explain some of the differences in the effects observed after injection of nephrotoxic sera, I believe that those indicated in comments (1) and (4) are perhaps more important determinants.

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SECTION III

Immunochemical Methods for Determining Homogeneity of Proteins and Polysaccharides

ASSOCIATE EDITOR—*Melvin Cohn*[*]

INTRODUCTION

THERE ARE 2 general methods for studying the homogeneity of proteins and polysaccharides: (1) physical methods—electrophoresis, ultracentrifuge, diffusion, solubility—which measure the effect of various forces on the molecule itself; (2) specific combining methods—activity (enzymatic, viral, hormonal) and antigenicity—which, to describe the properties of a given substance, utilize the reactions between different molecules. In special cases, the chemical methods of analysis can be included here, e.g., when the molecule under consideration has a known distinctive grouping, such as iodine in thyroglobulin, heme in cytochromes, etc.

These techniques for characterizing high molecular weight substances have become so refined that few purified proteins meet all the criteria of purity, for preparations apparently homogeneous by one test can be grossly inhomogeneous by another. This is not unexpected, since to a certain extent each method measures a different property of the molecule. The physical methods provide the only means for purification of proteins and polysaccharides, and the use of any particular technique selects those molecules whose homogeneity is assured only with respect to the property upon which the method depends. For example, a protein isolated by differential centrifugation would be expected to be homogeneous in the ultracentrifuge but might not be electrophoretically homogeneous. Therefore, the least sensitive way of detecting different molecules within a given preparation would be to use the technique employed in the isolation. Thus the pure protein or polysaccharide must be defined in terms of all the techniques used to study it. We would call a preparation homogeneous *when the differences between molecules are less than can be detected by any of the known methods.*

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[*] National Research Council Merck Fellow 1949–52, Pasteur Institute, Paris, France.

Although these methods are most often considered only as tools for the detection of impurities, they also lead one to define a fundamental question of biology and, at the same time, they provide the means to study it. The problem is to correlate physical and specific combining properties. Such a correlation involves the determination of which structural units are responsible for the specificity of a protein or polysaccharide, and of which physical properties are relevant or irrelevant (and to what extent) to the given specificity. These problems are very difficult to investigate and are largely unsolved. For instance, tetanus and diphtheria antitoxins, which are easily separated by certain of their specific combining properties, have never been distinguished by physical methods. Furthermore, diphtheria antitoxin, a single biologic entity as regards its qualitative ability to combine with toxin, can be associated with protein molecules of widely different solubility, mobility and sedimentation properties (2).

This section deals principally with an immunochemical method of characterizing proteins and polysaccharides, namely, the precipitin reaction. Although many proteins do not have known measurable biologic activity, almost all are antigenic, and this provides the basis for immunologic analysis. The most interesting and unique characteristic of this method is that one can prepare, at will, a specific test substance—antibody—for any given protein. Furthermore, this specific reagent enables the establishment of an independent classification of different molecules which are structurally more or less related. In addition, under certain conditions this analysis has extraordinary sensitivity, greatly exceeding that of the physical techniques for the detection of impurities.†

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† Throughout this section the word "impurity," when referring to immunochemical methods, will be taken to mean an antigenically distinguishable protein or polysaccharide in the substance under consideration. A cross-reacting precipitinogen would, therefore, also be considered an impurity.

In short, the interest in the antigen-antibody reaction as a research tool stems from the desire not only to detect traces of impurity in purified proteins, but also (1) to assay for a given antigen in mixtures of unrelated substances, (2) to reveal and characterize structurally related (cross-reacting) proteins and (3) to study certain alterations in a molecule that might occur during treatment.

The techniques of immunochemistry are not difficult and may easily be employed in any laboratory. Most of the methods and theory have been described by Kabat and Mayer (1), and the reader is referred to their excellent book for both the background and the bibliography of the subject. We do not intend to review the literature. Examples chosen are those most conveniently available to us, not necessarily the only ones. The section is designed essentially to provide details of techniques, but a discussion of the theory has been included because the interpretation of results is the more difficult aspect of this type of work. Because of the essentially empiric nature of immunochemistry, there are wide differences of opinion, of interpretation and of experience. These will be indicated, and the reader asked to judge for himself.

The review is divided into 3 chapters, each of which describes a particular aspect of immunochemical analysis.

I. Immunization of the experimental animal.

II. Fractionation of antibody containing γ-globulins from various species.

III. The techniques and analysis of the quantitative precipitin reaction: A, the reaction in liquid media; B, the reaction in gels.

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I. PRODUCTION OF ANTIBODIES IN EXPERIMENTAL ANIMALS

MELVIN COHN

THE PREPARATION of high titer antisera is the key step in the use of immunologic methods. Despite this, the technique of immunization has been the least developed phase of immunology. Two extensive analyses of the problem of antibody synthesis have appeared to which the reader is referred (2, 37).

The investigations of Freund and collaborators (10–16) suggest a new era of research which will lead to the production of reproducible preparations of antisera under absolutely controlled conditions. Since quantitative results on the immunization of animals are rare, we will discuss several practical aspects of the problem from the point of view of preparation of precipitating antibody to be used for studies on homogeneity of antigens.

FACTORS IN ANTIGENICITY AND THEIR SIGNIFICANCE

a) *The animal.*—Under ordinary conditions one will not find in the serum of animals antibody directed against their own proteins. There are certain exceptions, e.g., the organ-specific antigens—lens, testes. These antigens have not been isolated, and may not be proteins, but are antigenic in their own species (33). There are indications in the literature that antigens which cross-react with antibody against the proteins of a given species will, in certain cases, induce antibody formation in that species. For example, Uhlenhuth (43) found that a hare could be immunized with rabbit serum protein and produce an antiserum very highly specific only for rabbit protein, whereas if a chicken were immunized, the antiserum cross-reacted very extensively with rabbit and hare antigens. Another case is presented by Oudin who studied the cross-reaction between rabbit antisera to horse serum proteins, and the proteins of a wide variety of species, including man (35). He demonstrated that the serum albumin of man and of horse cross-reacted with rabbit antibody to horse albumin; yet, as is known, human serum albumin is a good antigen in the horse (17, 42). Other cases are to be found in the work of Landsteiner (33). It is not known, however, whether the antibody provoked by the closely related protein is directed against the common structural configurations or not. This important problem deserves further investigation, since it may enable the preparation of very specific antisera and may permit the study of specificity of antibody induction. However, in general, for investigations on homogeneity of antigens, one would avoid immunizing animals with their own or cross-reacting proteins.

Injection of a foreign substance into an animal does not guarantee an antibody response, for substances antigenic for one species may not be so for another. For example, isolated pneumococcus polysaccharide is antigenic for the mouse but not for the rabbit. This is an extreme case, but many substances have been described as good antigens in one species and poor antigens in another, and certain animals, e.g., guinea pigs, are considered poor producers of precipitin antibody while others, e.g., chickens, are considered good precipitin producers. In general, these statements are of limited significance for, by varying the route of injection or the dosage, by using adjuvants or by injecting the antigen coupled to another substance, the so-called poor producer of antibody may become a good one. For instance, if pneumococcus polysaccharide is injected as part of the whole bacteria cell or linked to a protein by artificial means, it becomes antigenic for the rabbit.

Most often, one does not have clean-cut, all-or-none reactions to antigens. Certain animals seem to respond better to one group of antigens than do others. For example, the horse appears to be a good producer of antitoxins and the rabbit a poor one. However, it is difficult to generalize, as no controlled experimental data are available in which the 2 species are compared. It is not possible to predict what substance will be antigenic in which animal. The question at present can be solved only empirically.

The animal's history is important. Previous contact with an antigen will often result in very high titer antisera, rapidly produced on injection of a recall dose. This anamnestic response has been used to prepare high titer diphtheria antitoxin in both human beings and horses by giving injections to Schick-negative individuals (6, 36). There does not seem to be an anamnestic response to polysaccharide antigens, a phenomenon discussed elsewhere (25).

Mature animals are usually better antibody producers than young ones, and therefore should be used whenever possible (47).

Almost nothing is known about the genetic factors involved in antibody production and their specificity. Investigations on the role of heredity in the ability to respond to given antigens would greatly aid in elucidating the problem of immunization and would certainly provide a wedge into investigations of the mechanism of antibody synthesis. For further information, see the work of Carlinfanti (4).

b) *The antigen.*—1) PURITY.—In general, a given antigen will elicit maximal antibody production when it is injected pure. If one immunizes with a mixture of several antigens, the probability of having antibody against any given one decreases. For example, isolated chicken ovalbumin is a fairly good antigen in the rabbit; however, whole egg white, which contains 60 per cent of this component, induces the production of much less antibody to ovalbumin. The major part of the antibody is directed against the very minor components of egg white (46). In certain cases, even with purified antigens, more antibody is produced against the minor component than against the major one (7). Results of this kind could be interpreted as meaning that the minor components, having greater antigenic power than the major one, tax the limited capacity of the antibody-synthesizing mechanism to produce antibody against them, thereby inhibiting or limiting the synthesis of antibody to the major component.

The response depends in part on the dosage of immunization (see p. 276). However, this interference by minor components does not always occur, for there are (*a*) cases in which addition of unrelated material enhances the antibody response, e.g., addition of staphylococcus toxin* (1), and (*b*) systems in which the antibody response to a given antigen is almost independent of its purity, e.g., diphtheria toxoid (26).

2) STABILITY.—No clear-cut data exist on the relation between the stability of a substance and its antigenicity. Yet it seems essential that the structural and combining groupings of the molecule be stable enough to reach the antibody-forming centers before being destroyed. Altered proteins are known to induce antibody of different specificity from that induced by the native form (34). A similar phenomenon may be obtained with proteins or polysaccharides immediately destroyed or denatured when injected in the blood stream, e.g., pepsin (40).

Antigens may be destroyed in the animals by various means, physical (maintaining at unfavorable pH and/or temperature) and enzymatic (proteolytic action). There are some indications that resistance of a protein to the animal's proteases may play an important role in determining its antigenicity. For example, the M protein of streptococci is readily destroyed by trypsin and is a very weak antigen, whereas the T protein is resistant to the protease and is an excellent antigen.

The question of stability may be more serious when the antigen is injected as an emulsion, which is absorbed slowly over a period of weeks from a sterile abscess, during which time the protein may be modified. This problem is probably less serious with most polysaccharide antigens which are generally very stable and in certain cases not known to be attacked by animal enzymes. This is reflected in the general property of polysaccharide antigens to induce a level of antibody formation which remains high for long periods, in contrast to certain antiprotein antibodies, the levels of which, after the end of immunization, decrease more rapidly (23, 31).

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* Sometimes irritants have a stimulating effect on antibody production.

A protein might be stabilized in vitro and therefore possibly as an antigen in several ways, e.g., addition of certain metal ions (19, 30) or of the substrate in the case of enzymes (3); furthermore, by treatment with formaldehyde, the antigenic structures might be stabilized (26). For preparation of antisera against easily denatured or nonantigenic proteins, the last method might prove very important. Treatment of toxins with formaldehyde to produce toxoids results in a loss of toxicity, but antigenicity remains unchanged. With respect to its antigenic properties, the resultant protein is much more resistant than the parent toxin to inactivation by heat or by extremes in pH. Treatment with formaldehyde might prove of great value in rendering weakly or nonantigenic proteins like the M substance antigenic. For example, when papain, which is not antigenic for the guinea pig, was treated with formaldehyde, it lost enzymatic activity but induced formation of antibody which neutralized papain activity (38, 48).

Holt (26) has described a method of formalinizing diphtheria toxin that seems to be generally applicable.

There are certain groups of substances considered non- or weakly antigenic. Low molecular weight proteins (protamine, gelatin, lysozyme) (39, 44) and glycoproteins (ovomucoid) (45), chondromucoid (9, 18) have been said to be weak antigens. It is difficult to explain these findings on any single basis. The poor antigenicity of glycoproteins might be a phenomenon specific for the rabbit, and the behavior of low molecular weight proteins might be accounted for in terms of their rapid excretion[†] or destruction. Use of adjuvants or treatment with formaldehyde might render these substances highly antigenic. In the case of lysozyme, an enhanced antibody response in rabbits has been obtained with use of adjuvants (39).

3) CHOICE OF EXPERIMENTAL ANIMAL.—The rabbit, chicken and horse are most useful for preparation of antisera. However, in certain cases, the guinea pig, dog, goat, sheep and fowl may be more suitable. In most circumstances, the rabbit is the animal of choice because it has been well studied and is convenient to handle. The

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[†] La Presle, working in Grabar's laboratory at the Pasteur Institute, Paris, has some preliminary evidence which shows a neat correlation between antibody level and renal excretion of several antigens. Those substances rapidly excreted, demonstrated by analyzing the urine, are weaker antigens.

chicken is a good producer of precipitating antibody and is extremely hardy. However, for quantitative work, its serum should be fractionated because of an apparent nonspecific co-precipitation of certain α-globulins (8). The horse, the only known producer of flocculating-type antisera‡ (see p. 323), is extremely useful for antibody production, but good facilities for injection and upkeep (as well as large amounts of antigen) must be available, thus limiting its use.

In the case of toxic antigens, apart from use of formaldehyde to render them nontoxic, it is sometimes possible to immunize a relatively resistant animal, i.e., urease in the hen (41).

4) PREPARATION OF ANTIGEN.—For most proteins whose antigenic properties are unknown, the adjuvant technique of Freund *et al.* (14) is most convenient. The antibody response is often greatly enhanced. This procedure has the advantage of using less antigen and fewer injections. The Freund technique follows.

For the EMULSION, materials include: 8.5 vol of Bayol F, a light paraffin oil (Stanco Distributors, New York) with 10 mg heat-killed Mycobacterium§ per 100 ml; 1.5 vol Arlacel A, a mannide mono-oleate (Atlas Powder Co., Wilmington, Del.); 10 vol of the antigen in buffer.

The Bayol F and Arlacel A can be sterilized by autoclaving; the antigen can be preserved with Merthiolate 10^{-4} by weight or sterilized by filtration. The heat-killed (70 C, 30 min), dried, acid-fast organisms, e.g., *Mycobacteria tuberculosis* or *butyricum*, are incorporated in the Bayol by grinding bacteria and oil together in a mortar.

The mixture of Bayol F and Arlacel A is made, then the aqueous phase added slowly during mixing. A Waring Blendor, with speed rheostat controlled, may be used. If possible, the Blendor should be kept cold to prevent overheating of the emulsion. With the small metal container supplied for the Blendor, as little as 5 ml of solution of antigen can be emulsified. If smaller quantities are to be prepared, or if a Blendor is not available, the mixture can be worked up and down in a syringe without needle, ground in a mortar or stirred with an electric-motor-driven bent glass rod. To test the emulsion, a drop at the end of an applicator stick is allowed to fall about 5 cm onto the surface of water. If the drop remains perfectly formed and does not spread out over the surface, the emulsion is ready for injection.

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‡ In brief, the reaction of flocculating antibody is characterized by the solubility of the antigen-antibody complex in excess antibody and a broad equivalence zone, in contrast to precipitating antibody which forms insoluble antigen-antibody complexes in excess antibody and shows a narrow equivalence zone.

§ It should be pointed out that lipid extracts of Mycobacteria and even cholesterol itself have been shown to have an augmenting effect on antibody formation (13).

Mycobacteria often augment the antibody response. Although one must be careful, when dealing with antigens from bacterial sources, to verify that no antigens of the Mycobacteria cross-react with the new antigen, it is advisable to immunize several rabbits with and without the Mycobacterium to observe its effect.

The QUANTITY OF ANTIGEN for injection by the Freund technique depends on whether the resultant antibody is to be used for assay purposes or to detect impurities. The production of antibodies against minor components is favored by giving massive doses over a short period of immunization, e.g., 50–100 mg antigen/rabbit and bleeding after 3 weeks. Smaller doses, 0.5–5 mg antigen/rabbit, and 6–8 weeks of immunization favor the production of antibody against the major component. We have had excellent antibody response in rabbits, guinea pigs and monkeys with single injections of as little as 0.100–0.500 mg diphtheria toxoid (6), ovalbumin or β-galactosidase (5). Grabar *et al.* (21) noted an excellent antibody response in rabbits with as little as 0.1 μg typhoid O antigen. In man, Heidelberger *et al.* have shown that as little as 30–40 μg pneumococcus polysaccharide is actively antigenic (25), and as a recall dose in man, 0.4 μg diphtheria toxoid can provoke an enormous response (6) of the order of 10^6 molecules of antibody for each molecule of antigen injected. For the horse, larger doses might be necessary, as much as 2–5 g of antigen/500–700 kg animal.

Several other carriers of antigen have been described, of which protamine, aluminum hydroxide and aluminum phosphate appear to be the most useful. Holt (26) has described the use of the latter 2 substances for immunization against diphtheria toxoid (see his book for description of the properties and preparations of these carriers). About 20–30 mg of antigen per 3 kg rabbit, divided into about 20 injections, is the usual dosage with this technique.

In the case of certain POLYSACCHARIDE ANTIGENS, immunization of the rabbit is carried out by injecting whole organisms. The pneumococcus polysaccharides can serve as an example. The organisms may be grown on neopeptone meat infusion broth, concentrated by centrifugation and resuspended in physiologic saline in a concentration of about 0.05 mg bacterial nitrogen/ml. They are then heat-killed at 65 C for 30 min in a water bath or treated with 0.2–0.5 per cent Formalin. Since the polysaccharide of the gram-negative form loses antigenicity, they must be checked by Gram stain before injection. Freund and Behan report that rabbit serum against the polysaccharide of type III pneumococcus can be prepared with living pneumococci more readily than by using bacterial cells killed by the usual methods (Formalin, flash heat, etc.) (14).

5) ROUTE AND SCHEDULE OF INJECTIONS.—With most animals, and with the antigen prepared in adjuvants, several techniques are possible. In general, a single injection of the antigen emulsion is all that is necessary before the test bleeding. For rabbits, the most satisfactory site of injection is the space behind the scapula, because it cannot be irritated by the animal. For chickens and guinea pigs, intraperitoneal injections are best, although the subcutaneous route is also effective. Three to 4 weeks after the beginning of immunization, a test bleeding is made. If the titer is not high enough (see p. 279), another injection with or without adjuvants often produces an enhancing effect, and further test bleeding is made 3 weeks later. If antibody is formed, its titer in the serum is usually sustained at a high level for many months and at a lower level for several years (16a). If no antibody is found after this, the other suggestions already discussed can be tried.

For antigens adsorbed on gels, in solution or in bacterial suspensions, a series of intravenous injections followed by subcutaneous or intraperitoneal injections is most often used. In a typical schedule, the animal is given 1 intravenous injection a day for 4 days and allowed to rest for 3 days. This may be continued for 4 weeks. To avoid danger of anaphylactic shock, it is sometimes necessary to change the route of injection, and after the 1st week the antigen preparation is given subcutaneously or intraperitoneally.

Another variation of the schedule is to give injections daily 5 times a week, the 1st subcutaneously (to desensitize and protect against anaphylaxis) and the following 4 intravenously. It is well to increase gradually the amount injected, starting with 1 mg total antigen/injection and increasing the dose to 5 mg.

The experience of workers with the methods of immunization differs, and each laboratory holds strongly to its own techniques. I have described the Freund technique in detail because it has proved successful with a variety of antigens and because very small amounts of antigen are necessary for maximal response—an important factor in work with precious materials.

However, Kabat and Mayer prefer to use repeated intravenous injections of alum-precipitated antigens for rabbits, because they have found much higher antibody response with ovalbumin, human serum albumin, γ-globulin and chymotrypsin as antigens. Their technique is described in their book (34). If sufficient of an untried antigen is available, it is recommended that 2 groups of animals be used, 1 with alum-precipitated antigen and the other with adjuvants. For guinea pigs, the latter technique is preferable.

Many observations have been made that the route of injection is important in the production of antibody (reviewed by Grabar (20)). From available evidence it seems that for the horse, the antibody response to protein antigen is minimal when intravenous injections are used. This is not true for polysaccharides. For other species, although the route of injection is probably important, the data do not justify practical conclusions regarding the production of antisera. The reader is referred to a series of fine investigations on the antibody response of rabbits and guinea pigs to diphtheria toxoid, as a function of dosage, route of injection, carrier of antigen and secondary response (15, 16, 26–29).

Careful study of all the available information on methods of immunization for commercial production of antibodies in horses shows that many different procedures are used successfully. The antigen in solution adsorbed on alumina gels or in adjuvants is injected by combination of all known routes—intravenous, intraperitoneal, intramuscular and subcutaneous—with total quantities of antigen administered ranging from 1 to 5 g in horses weighing 500–700 kg.[‖]

There is no general procedure, as far as I know, which can be recommended with certainty for the production in the horse of *flocculating antibody* to untried protein antigens. The data on immunization to diphtheria toxoid are not generally applicable because most workers choose Schick-negative horses and take advantage of the booster response. For serum albumin, Gitlin *et al.* (17) described a procedure beginning with alum-precipitated antigen and finishing with adjuvants. In its essentials, their technique has been repeated by Grabar at the Pasteur Institute, with the same antigen in the horse, and very high titer flocculating antibody was obtained. Possibly their immunization program is generally applicable. I have had some experience in immunizing large animals, donkey, goat and sheep, with chicken ovalbumin, human γ-globulin, atoxic diphtheria proteins and β-lactoglobulin. Responses were excellent when the procedure outlined below was followed. These animals produced precipitin type antibodies, and one cannot be certain that the procedure will carry over to the horse. However, there is sufficient chance that it will be useful and successful in immunizing a horse to an untried protein antigen to warrant its inclusion here. The dosages mentioned below are double those actually used by the author in the afore-mentioned animals.

Prepare the antigen in adjuvants with Mycobacteria, if possible. Give a subcutaneous injection each week, starting with 50

[‖] This information was obtained through the kindness of many drug houses and institutes producing antitoxins.

mg total antigen and increasing it 10 mg a week for 8 weeks.* Make test bleedings and examine serum for nonprecipitating as well as precipitating antibody. Presence of nonprecipitating antibody in the early stages of immunization is a good sign that a flocculating antibody will appear (9, 33). If no response is found, rest the animal for 1 or 2 months and repeat immunization. If antibody is formed, continue injection every 2 weeks with 150 mg antigen, making frequent test bleedings to see if the titer increases. For polysaccharide antigens, the intravenous route is preferable, with a schedule similar to that used for the rabbit, except that a dose of 20–50 times as much antigen is recommended.

6) TEST BLEEDINGS AND COLLECTION OF SERUM.—The test bleedings are extremely important and should be carried out carefully (for techniques of bleeding animals, see Kabat and Mayer (32)). Before injections are given, a control test bleeding must be made, and it should be shown that the given serum of the animal species does not react nonspecifically with the antigen to be studied.

After immunization, the antisera are screened by a ring test. The antigen in buffer solution (about 0.3 ml) is carefully layered over the antiserum (0.3–0.4 ml) in a narrow tube 3–4 mm in diameter. Appearance of turbidity or precipitation at the interface constitutes a positive result. Sera giving a negative reaction can be rejected. The precipitating sera can then be tested semiquantitatively. To 1 ml of each serum add about 100 μg of antigen. The precipitate is centrifuged off after 1 hr at 37 C. Another 50 μg of antigen is added to the supernatant and gently mixed without disturbing the precipitate. If further precipitation occurs after 1 hr at 37 C, the process is repeated until further addition of antigen causes no precipitation. The mixtures are allowed to stand overnight in the icebox (0–4 C), the precipitates are washed and their nitrogen contents determined (pp. 301 ff.). In ordinary circumstances, antisera containing less than 200 μg antibody N/ml should not be used.†

Animals showing good antibody levels should be exsanguinated after a fast of 18 hr to obtain sera free from dissolved lipids which separate on standing. It seems to this worker that prolonged immunization (6–12 months) and resultant frequent bleeding of the animal should be avoided because (1) the chances of having antibody production against minor impurities increase, (2) prolonged immunization may lead to a broadening of the equivalence zone and increasing ratio of antibody/antigen (24), and (3) in general, long-continued immunization favors the production of sera with less specificity. For the study of homogeneity, these effects would complicate the analyses and make any possibility of interpreting the ratios (antibody/antigen) in terms of molecular weight meaningless. However, for certain purposes, such as use of antiserum for assay and with very pure antigens, the continued immunization of rabbits offers no difficulty and in fact allows a saving on animals.

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* The use of subcutaneous injections is indicated also because some preliminary evidence has been presented that, with proteins, this route favors the production of flocculating antibody (42).

† With defined systems, an alternative for screening precipitin-type antisera is to add 10 and 25 μg antigen N to 1 ml portions of serum and, after incubation at 5 C overnight, separate the supernatants by centrifugation. These supernatants are treated to determine whether there is antigen or antibody excess (p. 307). If there is antibody excess after precipitation with the 25 μg N, the antiserum is probably good. If this supernatant has antigen excess but the supernatant to 10 μg N has antibody excess, the animal is further immunized. If the supernatant with the 10 μg N added shows antigen excess, the animal probably will not respond sufficiently to make its further use profitable. The choice of quantities of test antigen is variable, depending on its *R* value (see p. 308).

For most work, I believe that sterile technique is not necessary and is, in fact, cumbersome. The blood is collected in 50 ml Lusteroid plastic tubes and allowed to clot at room temperature. The clot is then separated from the wall (ringed) with an applicator stick and the tube placed at 37 C for 1 hr to let the clot retract. The serum is separated in an angle centrifuge for 1 hr at 5,000 rpm, collected and pooled.‡ The serum is allowed to stand overnight in the icebox and recentrifuged. Merthiolate 10^{-4} parts by weight and 0.2 per cent phenol are satisfactory preservatives provided they do not affect the antigen.

Comment by E. A. Kabat

It is the invariable practice in our laboratory to collect and process bloods sterilely and to add preservative. Much of the analytical work is also done with sterile glassware except for analytical pipets which cannot be heated without risk of altering their calibration. However, cotton plugs are put into the mouth end before use to avoid gross contamination. These precautions are essential in working with small quantities of antibody when tubes are allowed to remain in the refrigerator for a week before analyses are carried out and especially when repeated set-ups are made on the supernatant. It is also essential when sera are to be used for passive transfer test in human beings; for such studies the use of 0.4 per cent phenol is even preferable since it has considerable anesthetic effect on the skin.

Plasma is not very satisfactory as a source of antibody, because fibrin precipitates tend to form. The serum can be satisfactorily kept in solution or, in the case of γ-globulins, can be stored at −10 C as the lyophilized powder over P_2O_5 in vacuum. For human sera, Heidelberger and Di Lapi (22) present evidence that the best way to conserve the antibody is to store it in the frozen state at solid CO_2 temperature or simply in solution at 5 C.

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‡ Use of plastic tubes instead of glass is advantageous, since it allows centrifugation at higher speeds with slightly greater yield of serum. One must be careful, with certain species as the dog, that this speed of centrifugation does not cause hemolysis due to destruction of red blood cells.

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II. SEPARATION OF ANTIBODY-ACTIVE PROTEINS FROM VARIOUS ANIMAL SERA BY ETHANOL FRACTIONATION TECHNIQUES

HAROLD F. DEUTSCH, *University of Wisconsin*

IT IS OFTEN more desirable to study the chemistry of immune reactions with the "so-called" γ-globulin fractions than to use the whole serum. This allows the investigator to avoid or regulate the effects of non-γ-globulin factors such as complement and lipids which are known to react with antigen-antibody systems. Furthermore, after separation of the γ-globulins from weak immune serum, an antibody system of much greater potency can be reconstituted. The separation of the antibody from such entities as serum proteases also makes it possible to store solutions of these proteins with less danger of deterioration than is the case with whole serum.

Although reasonably good antigens may be obtained, the preparation of pure antibody is difficult and somewhat controversial. A part of this difficulty is concerned with variations in mass and charge of the antibody molecules in serum. Thus antibody fractions of varying solubility, mass, electrophoretic and biologic properties may be obtained from the γ-globulin fraction of a given serum. However, preparations showing reasonably uniform electrophoretic properties may be readily obtained from immune serum, and while they in general usually contain less than 10 per cent specifically precipitable protein they do represent material devoid of the other recognized electrophoretic entities of the serum.

The aqueous-ethanol fractionation techniques of Cohn *et al.* (3) have proved very suitable for the separation of such material from immune serum. They have many advantages over neutral salt fractionation methods and consequently we will deal only with separation of antibody-active protein system by aqueous ethanol. We are thus also neglecting a consideration of the subject of antibody preparation by the dissociation of specific precipitates.

Development by Cohn *et al.* (4) of more specific fractionation techniques which use metals as specific complexing agents will undoubtedly provide better techniques for antibody separations in the future. However, their applications to various animal sera will entail extensive investigation before practical procedures can be developed.

The variables in the ethanol type of fractionation are the alcohol concentration, ionic strength, pH, temperature and protein concentration. Their effects will be treated briefly and individually as regards their application to antibody fractionation.

ALCOHOL.—Ethanol has been most widely used, although other alcohols or water-miscible organic solvents of low dielectric constant may be employed. However, it must not be construed that a given concentration of ethyl alcohol is synonymous in its precipitating effect with another alcohol. Ethanol concentrations are usually expressed in volumes per cent or in mole fractions. The volume contraction resulting upon the addition of ethanol to an aqueous system may be neglected in the usual fractionation. Thus if 10 vol of 95 per cent ethanol is added to 15 vol of a 1 per cent protein solution the ethanol concentration is calculated as 38 per cent or as 0.152 mole fraction.

Ethanol under certain conditions is a good denaturing agent. Although various proteins show markedly different labilities in this respect, the antibody-active proteins are readily inactivated in the presence of alcohol if adequate temperature conditions are not maintained. The ethanol solutions are always precooled (−5 to −20 C) before addition to the protein system. The latter is cooled to near its freezing point, and in practice it is often desirable to have a small amount of ice in the system before the addition of ethanol. The temperature of the protein solution is lowered as the alcohol is added, usually being maintained within 1° of its freezing point until −5 to −8 C has been reached. At this temperature the antibody-active proteins of serum in alcohol up to ethanol concentrations of 40 per cent appear to show little denaturation as judged by the criteria of biologic activity and solubility in dilute salt solutions.

When adding alcohol to an aqueous protein system it is best to start with less concentrated reagent. In practice, a 50 per cent solution of ethanol is added until a concentration of 15–20 per cent has been obtained, and further additions may be made with 95 per cent ethanol. Use of the more concentrated alcohol obviates the obtaining of unnecessarily large volumes, but if this is not a consideration, more dilute solutions of alcohol may be used with somewhat greater safety. Obviously the rate of addition, the efficiency of stirring and the effectiveness of the agency for lowering the temperature during the ethanol addition are important factors in the fractionation. The temperature must fall as alcohol is added to an aqueous system, and the addition should not be carried out in such a fashion as to cause massive local precipitation with the inclusion of much protein that should be soluble at the given precipitation step.

A point of some interest is the possible deleterious effects of ethanol. The γ-globulin fractions of various animals have been found to contain varying amounts of material which appears to be approximately twice the molecular weight of the major portion (15). However, freshly fractionated (13) or electrophoretically separated (14) material appears to contain little or none of this heavier component. Cann *et al.* (2) have found that γ-globulins prepared by electrophoresis convection behave similarly. Also, Heidelberger *et al.* (9, 12) have shown that horse antiprotein serum, when delipidated or fractionated by ethanol, may give material of apparent higher antibody titer as measured by quantitative precipitin techniques. This may be due to aggregation of antibody-active and immunologically inactive protein in the γ-globulin fraction to give material which still reacts immunologically. Such considerations need further investigation and should serve to remind the investigator that fractionation conditions may lead to the production of material and subsequent immunologic reactions which have no counterpart in nature.

HYDROGEN ION CONCENTRATION.—The pH at a given alcohol concentration and temperature is not known with certainty. It is usually expressed as the value measured (1) at room temperature after dilution with weak NaCl solution, (2) at room temperature without dilution or (3) in the presence of the ethanol at a low temperature. The last condition is used when small amounts of valuable material must be fractionated and the amount of sample required for a pH determination cannot be discarded. The important consideration is to express under what conditions a given pH was obtained so that they can be met by another worker or in a future experiment.

The solubility of a given protein under stated conditions of ethanol, salt concentration and temperature is markedly influenced by the pH. It is usually at a minimum at the isoelectric point of the protein. Furthermore, at a given pH some proteins may show a net negative charge while others show the opposite. This may obviate some of the usual factors of solubility because of complex formation, particularly if the salt concentration is low.

TEMPERATURE.—The importance of temperature control in terms of protein denaturation in ethanol systems has been mentioned. A 2d important consideration is that of solubility. Usually pronounced decreases in solubility are mediated by temperature decreases, and vice versa. It is thus important to record the temperature at which a given precipitation was carried out, for variations in temperature from 3 to 5 C may often result in marked changes in yield or an increase in the amount of an impurity, depending on the particular fractionation step.

IONIC STRENGTH.—This represents an important variable in the ethanol fractionation; large changes in solubility may be effected as a result of relatively small changes in salt concentration. Moreover, specific anion and cation solubility effects are being recognized but have not been extensively explored. Our considerations will be limited simply to a consideration of ionic strength. It is assumed that the aqueous dissociation constants of the acids used more or less obtain in aqueous-ethanol solutions. The most commonly used salts in this type of work are those of acetate, carbonate, chloride and phosphate. In the usual pH range of fractionation (4–8), only the effect of the salt need be considered in the ionic strength (μ) calculation in which

$$\mu = \frac{1}{2} \Sigma mv^2$$

where m is the molality and v the valence of the ions in question. Since relatively dilute solutions are used the molarity may be readily substituted for the molality. It is apparent that the ionic strength contribution of any monobasic salt is the same as the molarity, but for a salt such as Na_2HPO_4 the ionic strength is 3 times the molarity. Calculation of the ionic strength necessitates the use of the foregoing equation, the pH and the Henderson-Hasselbach relation of $pH = pK + \log \frac{base}{acid}$. Thus if a known volume of protein solution is adjusted to pH 4.5 by the addition of dilute acetic acid or acetate buffer, the amount of the added buffer or acid which exists as sodium acetate may be readily calculated if the pH of acetic acid is known. The ionic contribution of the protein and acetic acid may be neglected. The ionic strength contribution to the system by the added salt is most readily determined in practice by construction of suitable graphs which show the relation between ionic strength and molarity at a given pH. In Figure 1 such a nomogram for acetic acid and phosphoric acid for the pH range 4–8 is given. The pH of acetic acid was taken as 4.73. Only pK_2, of the phosphate system, need be considered over this pH range and was taken as 6.7.

In initiating an antibody fractionation with serum it is considered that this system has an ionic strength of 0.15. Since in our practical preparation methods the 1st precipitation is carried out under conditions where the serum undergoes 8-fold dilution, the ionic strength now lies in the region of 0.02. The initial crude antibody precipitate is usually about 25 per cent protein and is suspended in water to give a 1–2 per cent protein solution for the final purification step. This results in an ionic strength of 0.0015–0.0008 before adjustment of the pH. After adjustment, the ionic strength may be calculated and then brought to the desired level before the alcohol addition by use of a buffer solution of the proper pH or of a neutral salt such as NaCl. Since the ionic strength is expressed in terms of the final precipitating condition, the diluting effects of the alcohol must be considered in this adjustment. The requisite amount of salt must have been added to the protein solution before the addition of alcohol in most fractionation steps.

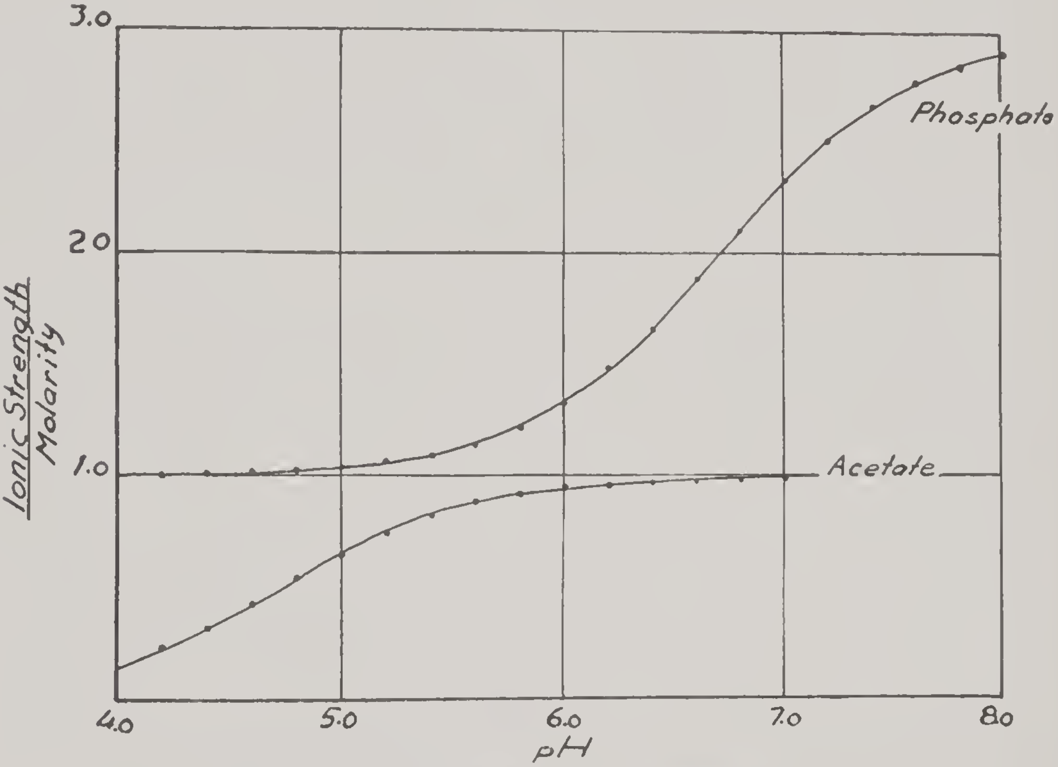


FIG. 1.—Ionic strength and pH relationship of acetate and phosphate buffers.

PROTEIN CONCENTRATION.—In theory it is desirable to fractionate extremely dilute protein solutions, but solubility considerations effect a more practical approach. If undiluted serum is used the liquid occluded in the precipitate will carry more contaminating protein than if a diluted system was utilized. A practical example of this follows.

Example: If a 1:4 dilution of serum is brought to a concentration of 25 per cent ethanol by the addition of 50 per cent ethanol, a 1:8 dilution of the serum has been effected. If for each 100 ml of a serum of 7 per cent protein concentration, 1.5 g of protein is precipitated and removed as 25 per cent protein paste, the solvent in the precipitate will contain approximately 0.03 g of soluble protein. Had the initial precipitate been removed by addition of an equal volume of 50 per cent ethanol to the undiluted serum, an equal amount of precipitate (1.5 g) would contain approximately 0.12 g of occluded protein. Actually the lower volume resulting in the case of the undiluted serum would probably result in more protein being occluded than indicated because of more protein-protein interaction, precipitation of more protein that would be soluble in less concentrated solution, etc. The actual protein concentration employed in a fractionation is thus an empiric consideration in light of our present knowledge and depends on such factors as type of serum, amount being fractionated, desired purity and nature of the material being isolated. In antibody fractionation work we have usually utilized precipitating conditions which employ approximately 1 per cent protein.

PROCEDURES

General considerations.—Any quantity of antiserum may be fractionated. It is imperative that a refrigerated centrifuge or a refrigerated room be available. Low temperature must be maintained during centrifugation. The precipitates obtained may be treated in various ways. If they are to be subjected to further fractionation they may be stored as the alcoholic pastes for some time at temperatures near their freezing points. It is, however, better to complete the fractionation as rapidly as possible and to avoid any such storage period in the form of an ethanol paste. During dissolving or suspending of any ethanol precipitate for lyophilization or further fractionation it is desirable to add to the pastes a small quantity of crushed ice before addition of the cold solvent. This tends to keep the temperature at a minimum during the suspending process. Small amounts of precipitate may be dispersed with a glass rod or a test tube type of homogenizer. Larger amounts may be suspended with a Waring Blendor or a similar device. Pastes representing the desired antibody fraction may be preserved for use in various ways. They may be suspended in water or dilute salt and then lyophilized. The use of a small amount of salt in this operation appears to give more stable protein preparations. The protein pastes may also be dissolved directly in an ice-salt or buffer solution and then dialyzed in the cold to remove the alcohol in the precipitate. This latter procedure tends to give more stable protein solutions than those subjected to lyophilization procedures.

The detailed procedures to be described for preparation of the antibody-active fractions of the serum of various animals are those with which we have had experience. Each system will be discussed briefly and individually and certain problems specific for a given serum will be treated.

The use of serum as starting material for preparation of γ-globulin is recommended because in the methods discussed here small amounts of fibrinogen are sometimes carried over to the γ-globulin fraction when plasma is employed. In solution this protein tends to flocculate slowly or clot out of solution, making it difficult to maintain a standard antibody solution suitable for quantitative immunochemical investigations. If plasma is collected it is recommended that the fibrinogen be removed before fractionation.

INITIAL PRECIPITATION.—The antibody-active proteins of all of the animal serums studied to date represent those electrophoretic globulin entities of the serum of highest isoelectric point. For this reason an identical initial precipitation step may be carried out for all the different serums. This consists in diluting the serum with 3 vol of water, adjusting the pH to 7.6–7.8, cooling to 0° C, then adding cold (-10 to -20 C) 50 per cent ethanol to a concentration of 18–25 per cent. This results in the more or less complete precipitation of all of the antibody-active protein (γ-globulins) with certain other proteins, notably β-globulins. This 1st precipitate is designated A in subsequent discussion. Secondary precipitation steps, usually in the neighborhood of pH 5, are now used to remove the contaminating proteins. This 2d precipitation step is referred to as B. The supernatant γ-globulins are now removed (as precipitate C) by adjustment of the pH to approximately 7.4 with $NaHCO_3$ and addition of ethanol to a concentration of 25 per cent.

γ-GLOBULINS.—It appears that all animal serums contain antibody molecules of quite different electrophoretic mobility. In the case of the human and horse systems they resolve fairly well in electrophoresis in pH 8.6 diethylbarbiturate buffer to give 2 families of antibody-active proteins which we have designated γ_2- and γ_1-globulins in order of decreasing isoelectric point. The γ_1-globulin, which in alkaline buffers has the approximate electrophoretic mobility of fibrinogen, has also been referred to as T-component, β_2-globulin, etc. We prefer to call this fraction γ_1-globulin because of the usual association of the term γ-globulin with serum proteins possessing antibody activity. In the cow and pig there appears to be a series of protein molecules in the γ-globulin fraction which, while showing marked electrophoretic heterogeneity, do not resolve into 2 components. Nevertheless it is possible to separate the latter antibody systems into fractions of quite different average isoelectric points. Again we have referred to these fractions as γ_2- and γ_1-globulins in order of decreasing isoelectric point. It is actually possible to separate a series of fractions of varying electrophoretic properties from a given serum. Although our main emphasis in this discussion will be to present conditions for the preparation of the γ-globulin of highest isoelectric point (γ_2-globulin), we shall discuss conditions for separation of the γ_1-globulin fraction in certain instances.

SUBFRACTIONATION.—Subfractionation of electrophoretically heterogeneous antibody protein is important, for preliminary investigations have disclosed that human and horse γ_2- and γ_1-globulin fractions show differences in antibody activity. Studies of the relationship of molecular mass, electrophoretic, solubility and other physical properties to various immunologic activities call for utilization of highly fractionated and carefully characterized antibody in which the molecules of any given fraction are as uniform as possible.

The conditions for separating the γ-globulins from the serum of various animals will be presented for each species. The reference after the name of each species indicates a publication describing fractionation of the discussed serum.

Rabbit (13).—Since this species is utilized so extensively in immunologic work, it will be used as an example of an animal whose serum antibody response appears to be largely in the electrophoretic fraction designated γ_2-globulin. The details of fractionating 100 ml of serum will be given. It will then suffice to present the fractionation of the other animal species in terms of conditions utilized at a given precipitation step or by reference to the analogous step used in the case of rabbit serum.

One hundred ml of serum is diluted to 400 ml with distilled water and pH measured at 25 C and adjusted to 7.7 (= 0.1) with $NaHCO_3$ or dilute acetic acid if necessary. Sera collected by centrifugation of clotted blood that has stood in the cold overnight usually possess the desired pH. The diluted serum is cooled to the freezing point and a small amount of ice allowed to form. Now 400 ml of 50 per cent ethanol, precooled to -10 to -20 C, is added slowly by means of a siphon as the diluted serum is being stirred as vigorously as possible without causing foaming. Temperature of the serum system is then brought down slowly to -6 C as the ethanol concentration is increased and maintained near this point until all of the alcohol has been added. No ice crystals should remain in the solution after sufficient alcohol has been added to lower the freezing point of the solution to -6 C. For small-scale operations such as this the diluted serum is placed in a glass beaker of sufficient size and immersed in an ice-salt bath or a cold ethanol solution to which small pieces of dry ice are added occasionally to lower the temperature to the desired level. Larger volumes can be cooled by insertion of a refrigerated coil into the material being fractionated. Less efficient is the cooling by conduction of the solution container that obtains on standing in a refrigerated room.

Fifteen to 30 min after completion of the ethanol addition, precipitate A of crude γ-globulins is removed by centrifuging under conditions that maintain a temperature between -5 and -7 C. The precipitate is suspended in 100 ml of water containing a few ice crystals. Cold 0.05 M acetic acid is then added to pH 5.1. After stirring for 10 min the pH is redetermined and adjusted to 5.1 if necessary. Ionic strength is now about 0.002. The precipitate (B) which forms at this point is then removed by centrifuging near 0° C. The clear supernatant solution which contains the γ_2-globulin is adjusted to pH 7.4 ($\pm$ 0.2) with cold 0.5M $NaHCO_3$. An equal volume of cold (-10 to -20 C) 50 per cent ethanol is slowly added and the temperature of the solution lowered to -6 C. After addition of the ethanol is completed the γ_2-globulin precipitate (C) is removed by centrifuging at -5 to -7 C. One of the several procedures indicated previously is then instituted to bring the precipitated protein to conditions under which it can be stored without danger of deterioration. Electrophoretic

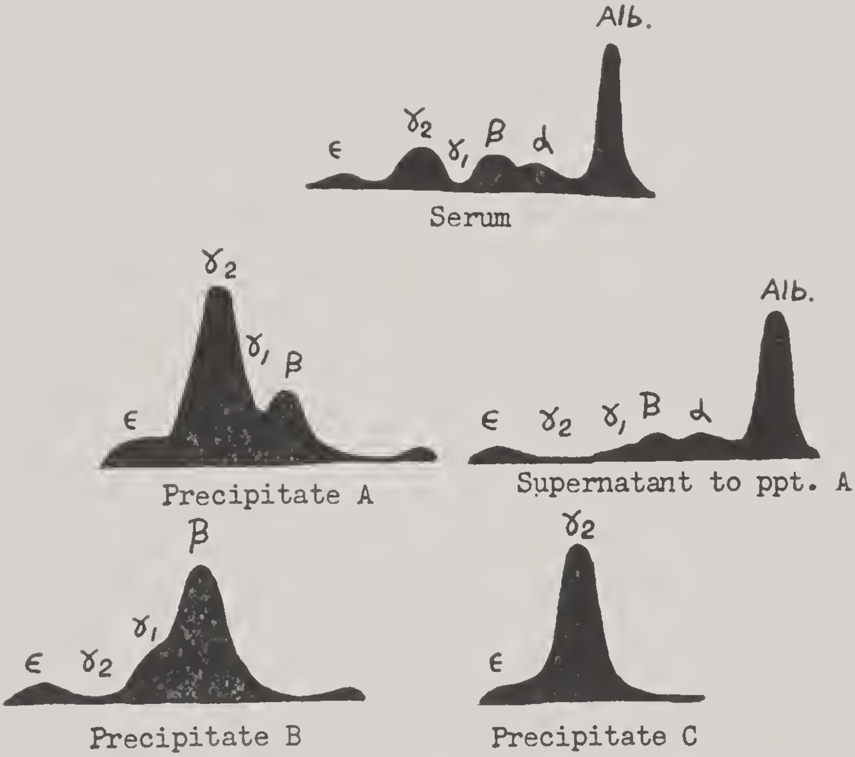


FIG. 2.—Descending electrophoretic patterns of rabbit serum and fractions in pH 8.6, 0.1 ionic strength, diethylbarbiturate buffer.

patterns in pH 8.6, 0.1 ionic strength, diethylbarbiturate buffer which indicate the course of the foregoing fractionation are shown in Figure 2.

The amount of γ_2-globulin obtained per unit volume of serum depends on the amount of this protein in the starting serum. This, as is well known, is a function of the degree of immune response of the animal(s) utilized. Conditions for recovery of the γ_1-globulin of rabbit serum have not been determined. The major portion of these proteins is precipitated at pH 5.1 (precipitate B).

Goat, rat, guinea pig, dog (13).—Only the γ_2-globulin entity of these serums has been prepared. The fractionation procedures differ from those used for rabbit serum mainly in the conditions used for removal of precipitate B. These are given in Table 1 together with the conditions utilized for the rabbit. The supernatants to precipitate B in the case of the rat and dog contain considerable amounts of γ_1-globulin. This is removed in admixture with some γ_2-globulin by a precipitation step at pH 5.6–6.0, μ = 0.005–0.01, at an ethanol concentration of 12 per cent. The γ_2-globulin containing supernatant to this precipitation step in the case of rat serum is essentially free from γ_1-globulin. All γ_2-globulin preparations from the dog contain 5–10 per cent of γ_1-globulin. An extensive investigation of conditions for removal of this material has not been successful. Guinea pig serum could not be fractionated to give γ_2-globulin preparations of high purity. Under the most optimal conditions found, a product which is approximately 80 per cent γ_2-globulin is obtained.

TABLE 1.—CONDITIONS FOR PRECIPITATION B FOR VARIOUS ANIMALS

ANIMAL	pH	ETHANOL, %	IONIC STRENGTH	APPROX. YIELD
Rabbit	5.1	0	0.002	75%
Goat	5.2	0	0.01	65%
Rat*	5.0	10	0.01	55%
Dog*	5.2	10	0.005	50%
Guinea pig	5.1	15	0.01	75%

* Supernatants to precipitate B for these species contain γ_1-globulin that must be removed by an additional precipitation step before precipitation of γ_2-globulin.

Man (7).—Precipitate A is suspended in water under the usual conditions and adjusted to pH 5.1. Ethanol, 50 per cent, is added to a concentration of 10 per cent. The temperature should be −2 C, and the ionic strength is minimal and approximately 0.0015 at this point. The precipitate (B) is removed by centrifugation and discarded. The supernatant proteins are then precipitated at pH 7.4 in the usual manner. This precipitate (C) consists almost entirely of γ_2- and γ_1-globulin with a great preponderance of the former. It can be subfractionated by the following procedure.

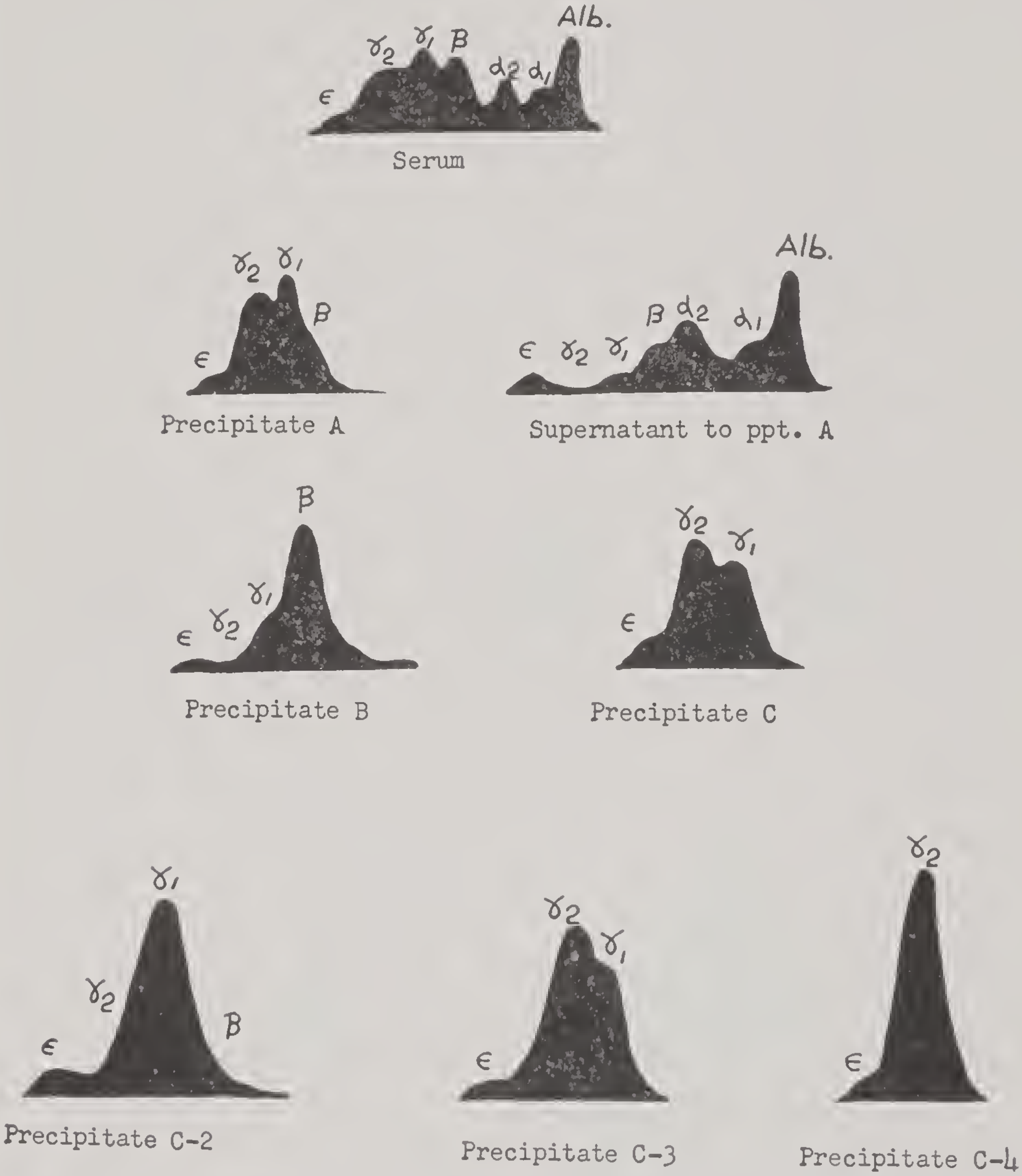
SUBFRACTIONATION.—The precipitate is suspended in water to give a concentration of approximately 2 per cent and dilute acetic acid is added to pH 5.1–5.3. The solution is stirred for approximately 15 min and any insoluble material is centrifuged off and discarded. The solution is then adjusted to pH 5.5 with Na_2HPO_4 and sufficient NaCl is added so that after the addition of 50 per cent ethanol to a concentration of 10–12 per cent an ionic strength of approximately 0.005 results. The precipitate formed under these conditions consists largely of γ_1-globulins but should be refractionated under the above-described conditions at pH 5.5 one or more times to prepare material of relatively uniform composition. The supernatant to the first pH 5.5 precipitate is adjusted to pH 7.4 with $NaHCO_3$ and 95 per cent ethanol is added to a concentration of 25 per cent. This precipitate consists largely of γ_2-globulin and can be separated from about 10 per cent of γ_1-globulin by the following precipitation steps. The precipitate is suspended to give approximately 2 per cent protein concentration, the pH is adjusted to 5.1 with dilute acetic acid and sufficient NaCl is added so that after the addition of 50 per cent ethanol to a concentration of 20 per cent an ionic strength of 0.01 results. The small precipitate forming is removed and discarded, the supernatant adjusted to pH 7.4 with $NaHCO_3$ and 95 per cent ethanol added to a concentration of 25–30 per cent. The precipitate consists almost entirely of γ_2-globulin.

It is to be stressed that the re-precipitation steps, while resulting in yield decreases, are necessary to obtain material containing only small amounts of the other γ-globulin fraction, i.e., if the isoelectric points of the molecules making up such material are to show very little overlap. Actually it is possible to separate a series of γ-globulin fractions from human serum showing a gradation of physical and immunologic properties (5).

Horse (6).—The commercial importance of horse antitoxin serum and the serum protein changes resulting during immunization make this animal an especially interesting species to study from an immunologic standpoint. A particularly interesting feature of horse immune serum of the antitoxic type is the large amount of γ_1-globulin.

Precipitate A is removed in the usual manner and brought into solution at pH 5.2 at minimal ionic strength (approx. 0.0015). Alcohol is added to 10 per cent concentration and the precipitate (B) formed is removed. The supernatant proteins are precipitated at pH 7.4 in the usual manner. This precipitate (C) consists of a mixture of γ_2- and γ_1-globulins, their ratio depending on the starting serum. Normal, weakly antitoxic and antibacterial serums show a higher amount of γ_2-globulin. Strong antitoxic serum gives a precipitate C that contains larger amounts of γ_1-globulin.

SUBFRACTIONATION.—To subfractionate precipitate C, the material is brought into solution at approximately 1 per cent protein concentration at pH 5.4 and minimal ionic strength (approx. 0.0015). The insoluble material (precipitate C-1) usually contains very little antibody and is discarded. The supernatant is adjusted to pH 5.8 and sufficient NaCl is added so that after addition of 50 per cent ethanol to 10 per cent concentration an ionic strength of 0.005 is obtained. The precipitate (C-2), consisting largely



Serum

Precipitate A

Supernatant to ppt. A

Precipitate B

Precipitate C

Precipitate C-2

Precipitate C-3

Precipitate C-4

FIG. 3.—Descending electrophoretic patterns of horse serum and fractions in pH 8.6, 0.1 ionic strength, diethylbarbiturate buffer.

of γ_1-globulin, is removed. The supernatant is brought to 20 per cent ethanol and precipitate C-3, consisting of a mixture of γ_2- and γ_1-globulins, is removed. The supernatant is brought to pH 7.4 and 95 per cent ethanol is added to a concentration of 25 per cent. The precipitate (C-4) consists largely of γ_2-globulins. When fractionating serum containing large amounts of γ_1-globulin it may be necessary to remove precipitate C-3 at an ethanol concentration of 25 per cent to prevent small amounts of this protein from being carried over into the next precipitate. Conditions have not been found by which the major portion of the γ_2- and γ_1-globulins can be separated into single fractions, each relatively free from proteins contained in the other fraction. Under the precipitating conditions employed, varying amounts of the γ_2-globulins have solubilities close to the γ_1-globulin, and vice versa. Individual serums show a great deal of variation in the yields, and the physical character of the fractions separated, particularly precipitate C-3, cannot be predicted without previous fractionation experience on a given immune serum.

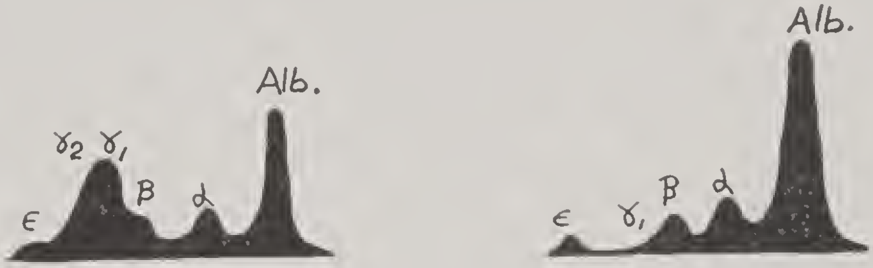
Electrophoretic patterns in pH 8.6, 0.1 ionic strength, diethylbarbiturate buffer showing the course of a typical horse serum fractionation are given in Figure 3. Unlike the freshly prepared γ-globulin fractions of other species, the horse proteins, when examined in the ultracentrifuge, show the presence of 10–20 per cent of material of approximately twice the molecular weight of the normal components (6). This material appears to be a product formed during fractionation, for examination of γ-globulins recovered by electrophoretic separation shows only the normal component of $s_{20w} = 7\,S$ (14).

Cow (10, 11).—The globulin fraction of this serum is represented by protein molecules which, like those of the other animals, show a wide isoelectric point range. They do not, however, tend to separate into discrete components, during electrophoresis, as is true for the horse and man. Two fractions of different average isoelectric point possessing antibody activity may be separated. The conditions described below tend to give approximately equal amounts of the so-called γ_1- and γ_2-globulin fractions.

Precipitate A is best removed at an alcohol concentration of 18 per cent. This material is separated from β-globulins by precipitation of the latter contaminants at pH 5.0, $\mu = 0.01$. The supernatant globulins are recovered in the usual manner as precipitate C. This fraction is then divided into approximately equal portions by adjusting a solution approximately 1 per cent protein to pH 5.8, $\mu = 0.01$ and ethanol 10 per cent. The supernatant to the precipitate (C-1) which forms is adjusted to pH 7.4 and ethanol

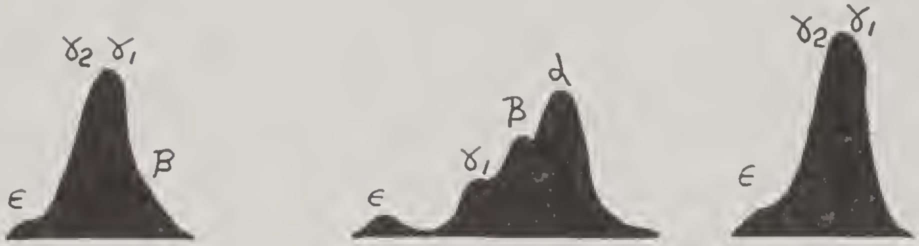
25 per cent. The higher average isoelectric point fraction (precipitate C-2) designated as γ_2-globulins is then removed.

The electrophoretic diagrams in pH 8.6, 0.1 ionic strength, diethylbarbiturate buffer which follow the course of this fractionation are shown in Figure 4. The γ_2- and γ_1-globulin fractions each contain considerable amounts of protein common to the other fraction; i.e., they show a pronounced overlap in isoelectric point distribution. The average isoelectric point and electrophoretic heterogeneity of either γ-globulin fraction may be altered by utilization of conditions at the C-1 precipitation step which tend to increase or decrease the amount of protein precipitated at this point. An increase in the amount of γ_1-globulin fraction precipi-



Serum

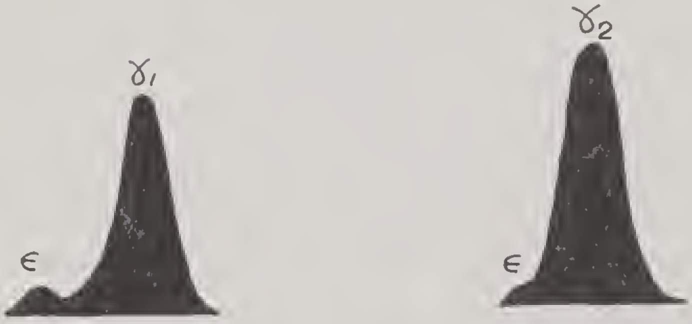
Supernatant to ppt. A



Precipitate A

Precipitate B

Precipitate C



Precipitate C-1

Precipitate C-2

FIG. 4.—Descending electrophoretic patterns of cow serum and fractions in pH 8.6, 0.1 ionic strength, diethylbarbiturate buffer.

tated, which may be mediated by increasing the ethanol concentration above 10 per cent will result in a small yield of γ_2-globulin of higher isoelectric point and decreased electrophoretic heterogeneity. Conversely, a γ_1-globulin fraction of lower average isoelectric point and heterogeneity may be recovered by decreasing the amount of ethanol used at the C-1 precipitation step.

Pig (1).—The γ-globulin fraction of the pig resembles that of the cow as regards the electrophoretic heterogeneity of the protein fractions that are obtained. In this case the γ_2- and γ_1-globulin fractions separated show a greater heterogeneity than is seen for the bovine systems. It is also more difficult to remove the β-globulins from the γ-globulin fraction.

Precipitate A is removed in the usual manner but at an ethanol concentration of 20 per cent. It is suspended to give a 1 per cent protein solution and pH is adjusted to 5.2. After stirring for 30 min, precipitate B is removed. The crude γ-globulins in the supernatant are precipitated with 25 per cent ethanol at pH 7.4.

Precipitate C is suspended in water to give a 1 per cent protein solution and adjusted to pH 5.6. Sufficient NaCl is added to give an ionic strength between 0.005 and 0.01 when 50 per cent ethanol has been added to a concentration of 15 per cent. The precipitate (C-1) consists of crude γ_1-globulins with 5–10 per cent β-globulins as impurities. It is further fractionated by suspending in water and removing a precipitate at pH 5.2, ionic strength 0.005 and ethanol concentration 5 per cent which consists largely of β-globulins. The supernatant γ_1-globulins are recovered by precipitation at an ethanol concentration of 25 per cent at pH 7.4.

The γ_2-globulins in the supernatant to precipitate C-1 are recovered at an ethanol concentration of 25 per cent at pH 7.4 and ionic strength approximately 0.01.

As in the case of the bovine globulins, the amounts and electrophoretic properties of the γ_2- and γ_1-globulin fractions may be altered by modification of the conditions giving rise to precipitate C-1.

Chicken (13).—The serum of this species is characterized on electrophoretic study in pH 8.6 diethylbarbiturate buffer by absence of a component corresponding in mobility to the mammalian γ_2-globulin. Two distinct γ-globulin fractions which we have also designated γ_2- and γ_1-globulins are present, but they have considerably higher mobilities than the proteins of similar biologic function have in higher animals. In addition, there appear to be protein(s) possessing the mobility of α-globulins in some antisera which can add on to antigen-antibody complexes even though they are unable to form precipitates with antigen in the absence of chicken γ-globulin (8).

Precipitate A is taken off under the usual conditions at pH 7.7 and 25 per cent ethanol. This material is then fractionated at pH 5.0, $\mu = 0.01$ and ethanol 10 per cent to give precipitate B. The γ-globulin-containing supernatant is then precipitated between pH 7.0 and 7.4 at an ethanol concentration of 25 per cent. The γ-globulins recovered at this step give asymmetrical electrophoretic patterns in pH 8.6 buffer and appear to be a mixture of γ_2- and γ_1-globulins. They differ from mammalian γ-globulins in giving a sedimentation constant of 7.8–8.0 S. The γ-globulin fractions also often contain varying amounts of serum conalbumin (transferritin) which may be readily recognized on ultracentrifugal analysis or by determining the iron-binding capacity of the γ-globulin preparation. The conalbumin can be removed by repeated precipitation of precipitate C at $\mu = 0.01$, pH 7.0–7.4 and an ethanol concentration between 20 and 25 per cent.

The nature of the α-globulin component which can enter into the antigen-antibody reaction is poorly understood. It is soluble at the conditions used to remove precipitate C. The differences in the appearance of specific precipitates formed by addition of antigen to whole serum and of γ-globulin fractions suggests that the α-globulin factor is lipoprotein in character. The ability of a factor in the α-globulin to add on to an antigen-γ-globulin system in this species to give markedly increased amounts of specific precipitate is an example of complications that can be avoided and controlled in immunologic work when one utilizes antibody preparations of the γ-globulins rather than whole serum.

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III. TECHNIQUES AND ANALYSIS OF THE QUANTITATIVE PRECIPITIN REACTION

A. Reaction in Liquid Media

MELVIN COHN

1. MATERIAL AND METHODS

WE WILL DISCUSS a quantitative method for following the reaction which results in the formation of a specific precipitate between a soluble antigen and an antibody. This insoluble precipitate is freed from nonreactive substances by centrifugation and washing with buffer or saline and the quantity of nitrogen precipitated is determined by a micro Kjeldahl method.

The particular variation which we prefer has a number of distinct advantages over other techniques. However, it is one of a dozen procedures and is presented only because it has not been discussed in full elsewhere.* It can be changed and simplified in several ways. The problems involved in carrying out this procedure are: (1) to avoid nonspecific precipitation; (2) to have the system in equilibrium, and (3) to separate completely the specific precipitates from the supernatant fluids.

Certain general features deserve mention. It is preferable, but not always essential, to fractionate the antiserum for the antibody-containing γ-globulin. This eliminates complement† (see Deutsch, this volume), certain lipids which separate on standing and, very often, nonspecific precipitation. The choices of conditions for the antigen-antibody reaction must be compatible with the stability of each reactant. For most cases, a pH close to neutrality and a salt concentration close to isotonicity are suitable. If the antigen is a protease, its possible action on the antibody must be considered and conditions should be chosen such that the enzyme has no significant activity.

There are several ways to begin the study of an unknown system and to evaluate these factors. The following procedures are sufficiently controlled and reliable, but they can be carried out in many ways.

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* If certain equipment discussed here is not available, see Kabat and Mayer (11) and Loiseleur (12), for detailed description of other techniques.

† In general, addition of complement to the specific precipitates will not specifically change results. However, in work with very small amounts of antibody, the problem is important and complement must be removed; for the technique, see (1). Another useful means of eliminating complement is to allow the sera to remain in the refrigerator until complement activity disappears.

A. SETTING UP ANTIGEN-ANTIBODY MIXTURES

a) *Conical tubes*‡ (Fig. 1).—These must be made of the highest quality pyrex glass to withstand repeated heating and centrifugation. They can be obtained calibrated to facilitate colorimetric reactions for determination of total protein precipitated. All operations up to the actual nitrogen determination are carried out in the same tube.

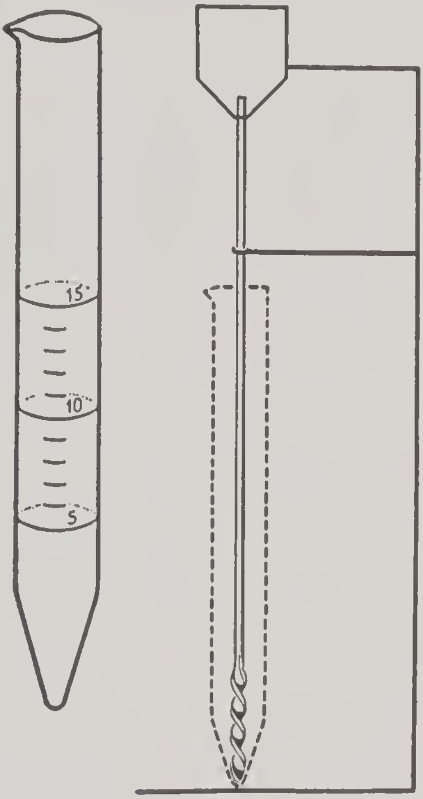

FIG. 1.—Conical tube and stirring apparatus for resuspending specific precipitates. Length of tube, 15 cm; width, 1.8 cm.

b) *Technique.*—Before carrying out the reaction, thoroughly centrifuge antigen and antibody at the same speed that will be used in later centrifugations. Choose a serum diluted to contain about 150 μg antibody in 1 ml, as determined by preliminary tests (p. 311). To a series of conical tubes add varying amounts of isotonic buffer (or 0.15M NaCl) in volumes calculated to give a final fixed volume between 2 and 4 ml. Within the limits of solubility considerations, the precipitin reaction is volume-independent. Increasing amounts of antigen (starting at 2 μg N for protein and 10 μg for polysaccharides) are then added, followed by the 1.0 ml fixed volumes of antibody solution. The latter should be added rapidly and the tube contents mixed immediately thereafter by rolling the tubes rapidly between the hands. This is done to avoid the Danysz effect (2).

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‡ Made on order by E. Machlett & Son, 218 E. 23d St., New York 10, and by Delplanque, 212 rue Saint Jacques, Paris, France.

One of the greatest hazards in carrying out the quantitative precipitin reaction is the possibility of nonspecific precipitation. This is particularly marked with crude bacterial extracts, proteins of very high molecular weight and proteins with isoelectric points at the extremes of pH. Nonspecific precipitation can sometimes be avoided by choice of conditions of precipitation or by fractionation of antisera. In certain cases it occurs only at protein concentrations much higher than those used in the specific reaction.§ As a check on nonspecific precipitation, controls must be set up, including the antiserum alone plus buffer, antigen plus normal serum obtained before immunization, and antigen plus unrelated antiserum. No precipitation should occur in these controls. If nonspecific precipitation cannot be avoided, a control with normal serum can be set up for each mixture, and this value subtracted from that obtained with antiserum. Results under these conditions are at best rough approximations.

The various mixtures should be set up in duplicate. In case of shortage of antigen or antibody, it is better to have double the number of different mixtures than fewer in duplicate.

Certain preliminary tests must be made to determine correct conditions for studying the reaction, in particular the effect of incubation and washing. Three different amounts of antigen, chosen on the basis of preliminary tests (p. 311), should be added to constant volumes of antiserum, such that the resultant systems are (*a*) in antibody excess, (*b*) near the equivalance point and (*c*) in antigen excess. Each of these should be prepared in octuplicate. In general, 4 incubation conditions can be tried, with 2 tubes at each point: (1) 4 C for 2 days; (2) 4 C for 5 days; (3) room temperature until precipitation occurs (2–3 hr) and 2–3 days at 4 C; (4) 40 C until precipitation occurs (1–2 hr) and 2–3 days at 4 C.

The tubes are centrifuged after the various incubations; 1 tube of each pair is washed 3 times and the other 5 times (see *B* below) and N content of the precipitates determined. Thus in 1 preliminary experiment the effect of incubation and of washing is evaluated. In most cases the precipitate is sufficiently insoluble so that no significant difference between 3 and 5 times washing will be noticeable. If the specific precipitate is appreciably soluble, washing must be minimal, and correction for solubility can be made.

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§ With certain substances such as ricin which react nonspecifically with serum proteins, estimation of antibody in specific precipitates is not reliable. Although only high concentrations of ricin alone will react with normal serum, specific precipitates of ricin with antibody, even with concentrations below that at which ricin visibly reacts with normal serum, appear to contain both antibody and nonspecific protein (54). In this case it would be interesting to know whether the isolated antibody-containing globulin also reacted nonspecifically.

Many factors are involved in the attainment of complete precipitation. It is more difficult to obtain reproducible results in the region of antigen than in the region of antibody excess. In cases of difficulty, incubation at 4 C for long periods (7-14 days) has given reproducible results. Often, however, for reactions involving more than 100 μg antibody N, maximal precipitation occurs after 2 days at 4 C and further incubation has no effect. These factors must be tested in each new system, since they may vary considerably. With certain antigen-antibody reactions, the same results will be obtained under any of these conditions, in which case it is preferable to incubate at 4 C for 2 days. In the presence of viscous solutions, e.g., bacterial extracts or high protein concentrations, preliminary incubation at high temperatures (37–45 C) is sometimes necessary before storage at 4 C in order to obtain equilibrium.

B. WASHING SPECIFIC PRECIPITATES

a) Apparatus.—1. Centrifuge. We use the International Refrigerated Centrifuge model PR-1 with no. 269 Mitat head. Eight tubes can be spun at 1 time at 3,000 rpm. Although the tubes fit loosely into the no. 320 Atab cups, the only necessary precaution is to turn the pouring spouts to the outside, since breakage might otherwise occur when the long tube, spinning horizontally, touches the head.

2. Stirrer.[‖] The specific precipitates can be resuspended in the washing solution with a small glass stirring rod drawn out to a point. However, in work with large numbers of samples or with voluminous precipitates, a power-driven stirrer is preferable. A fine spiral propeller of stainless steel (Fig. 1) turning at about 1,000 rpm is very efficient. It should be well guided so that it turns perfectly centered.

Comment by E. A. Kabat

Experience in our laboratory has led us always to avoid a procedure in which a stirrer or any other object was placed in the tube for washing specific precipitates. After the tubes have drained, the precipitates can be broken up by tapping the tubes vigorously; 0.5 ml chilled saline is added and the precipitates are uniformly suspended in this small volume. The additional volume of wash liquid is then added and the precipitates are

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[‖] There are certain objections to using a stirrer with polysaccharide precipitates in antigen excess, because they are somewhat gummy. If poor values are obtained, the precipitates may sometimes be resuspended by holding the tube at the neck with 1 hand and tapping the bottom, so as to set up a rapid revolving motion of the wash liquid.

dispersed uniformly by gentle twirling. By this procedure, the need for introducing a stirrer in the tube is avoided.

b) *Technique.*—The tubes are centrifuged at 0° C for 1 hr at 3,000 rpm for the 1st separation of supernatants, which should be absolutely clear. Even if a given tube seems to have no precipitate, it should be centrifuged and treated as the others because certain precipitates are very hard to see in the presence of serum. In some cases these supernatants remain turbid, but often they may be clarified by centrifugation at higher speeds (10,000 rpm in a Servall angle centrifuge), or in certain cases, if fractionated antisera are used, turbidity will be avoided. If a refrigerator room is not available, the tubes are kept cold in an ice bath. The supernatant is carefully poured off and saved, and the conical tube is allowed to drain upside down on blotting paper. Four ml of cold buffer or saline is added so that it washes down the tube wall. The precipitate is resuspended and finely divided with the mechanical stirrer or simple glass rod, and the stirrer washed down with another 1 ml of wash solution. The tubes are then centrifuged, and the operation is repeated 3 times. The recentrifugations can be made for 15 min periods.

C. DETERMINATION OF QUANTITY OF SPECIFIC PRECIPITATE

a) *Materials.*—1. Digestion mixture: Various solutions have been described—all quite good. The simplest is made by adding cautiously 20 ml of a saturated solution of aqueous copper sulfate to 1 l of concentrated sulfuric acid (Merck C.P., "low nitrogen"). The excess anhydrous copper sulfate settles out after several days, and the supernatant is used for the digestion process.

2. Potassium sulfate: Special low nitrogen analytical reagent (Merck).

3. Digestion rack: One of the most convenient is an electrically heated sand bath with temperature controlled by a rheostat in the external electric circuit. Figure 2 shows that used at Pasteur Institute. The heating elements (1,500 w) are covered with sand, and the conical tubes are placed through a support at a 45° angle into the sand so that only the conical part is below the surface.

4. Distillation apparatus: We prefer that made by Scientific Glass Apparatus Company, Bloomfield, N. J., because it is compact and constructed with all-glass connections.

5. Titration: Although most laboratories use indicator titration, electrometric methods are more precise and reliable and much less fatiguing. We have used the quinhydrone electrode successfully but are now using the Beckman pH meter model G with shielded glass calomel outside electrodes no. 11-305-70 and 8970-90. The

sample is mixed with a magnetic stirrer, which does not affect these electrodes. Titration is carried out with a Scholander buret (3) filled with acid, so that 100 divisions of the micrometer equals 20 μg N. This standard acid is prepared by dilution of a weighed amount of constant boiling HCL (4).

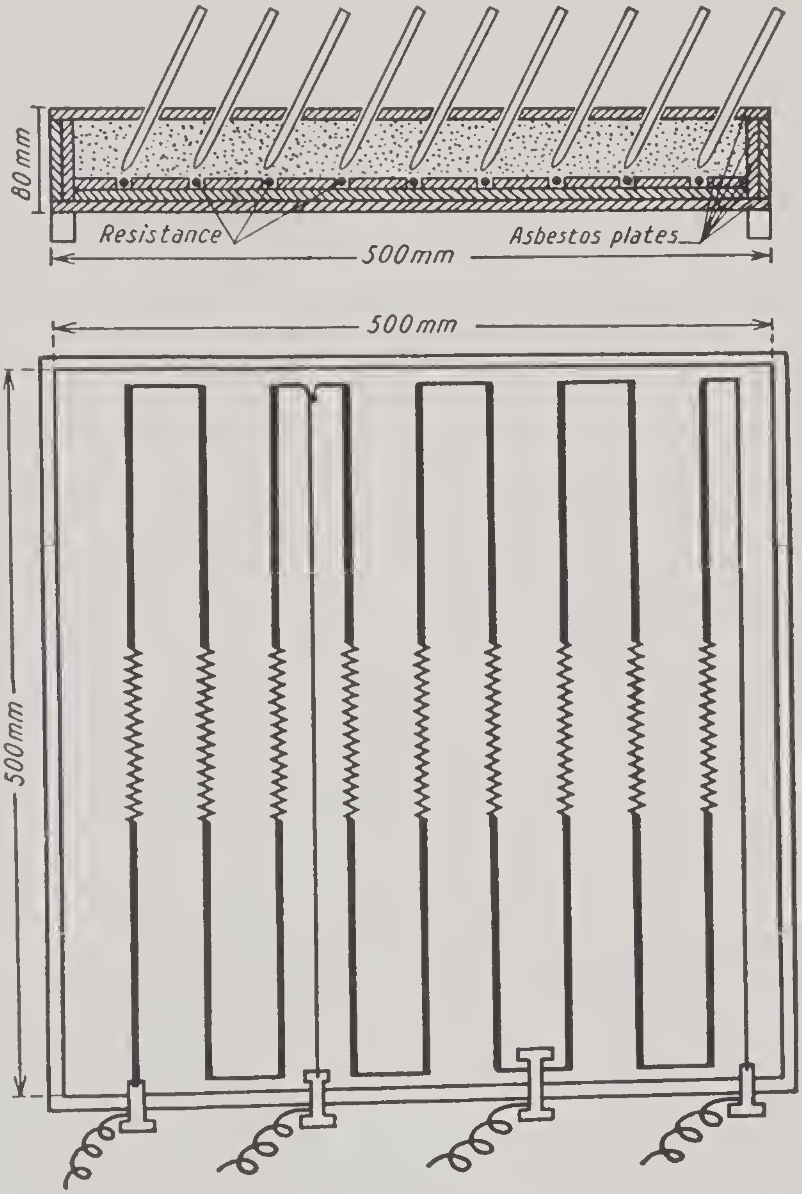

Fig. 2.—Diagram of electrically heated digestion rack (sand bath) for use with conical tubes. Any convenient electric circuit can be used; that shown here is only a suggestion.

b) *Technique.*—The quantity of specific precipitate is best determined by N estimation. For initial studies with unknown systems, no other method is satisfactory. When the reaction is well defined and when the antigen or antiserum is to be used for assay purposes, the rapid, simple and often more sensitive absorption and density determinations are of great value (5–10).

Five-tenths ml of the digestion mixture is added to each tube, plus 250 mg of potassium sulfate. The latter is conveniently added with a little scoop made out of brass rod which will just hold 250 mg. The tubes are transferred to the digestion rack. No boiling stones are used, and the acid, without ebullition, refluxes gently about halfway up the tubes. In general, after about 3–4 hr of reflux, digestion is complete. The time of complete reaction should be checked when working with a new system.

The conical tube contents are transferred to the distillation apparatus by repeated washing with a total of 30 ml water. Then 3 ml of a saturated NaOH solution is added and distillation begun into 3 ml of a saturated solution of boric acid in a 50 ml beaker scratched to indicate volumes equal to 20 and 25 ml with a diamond point pencil. The exit of the distillation apparatus is placed below the surface of the boric acid solution, and after the volume of distillate reaches the 20 ml mark the beaker is lowered so that the opening is out of the liquid and distillation continued to the 25 ml mark in order to rinse the condenser. Distillation is stopped. The tip of the refrigerant column is rinsed with 2 ml water and the sample is ready for titration. For titration, the acid is added to pH 5.0 from the Scholander buret whose tip is placed just below the surface of the liquid. This determination is quite accurate with N concentrations between 10 and 150 μg, which is a sufficient and convenient range for immunochemical work of the type discussed here.

D. TESTS OF SUPERNATANTS

For interpretation of results it is essential to know whether there is excess of antigen or antibody in the supernatants. This is carried out qualitatively as described below.

On the basis of the quantitative precipitin reactions, a general idea of the sensitivity of the test for excess antigen or antibody can be obtained. However, it is important to know the sensitivity with precision, and this is done by adding to fixed volumes of antiserum (containing about 100 μg antibody N) increasing amounts of antigen, 0.1, 0.5, 1.0, 2.0, 5.0 μg. These mixtures are incubated as for the original reaction and centrifuged. A very small precipitate is most easily detected as a centrifuged pellet. The concentration of antigen which is just detectable as precipitate is the limit of our tests. This value is generally between 0.5 and 1.0 μg antigen. The test for sensitivity of antibody detection is carried out by adding dilutions of antiserum corresponding to between 3 and 10 μg antibody N to 1 and 5 μg antigen. The mixture in which the least amount of antibody is detectable decides the amount of antigen to be used to test for antibody excess. Since detection of antigen excess is more sensitive than that of antibody excess, $^1/_2$ of the supernatant is used for the former and $^3/_4$ for the latter. The method of analysis depends on the results of the preliminary tests; one uses the conditions which give maximum sensitivity.

In the case of antigens which have measurable specific properties, i.e., enzyme, virus, hormone or chemical activity, distinct grouping as heme, distinct absorption as nucleic acid, such properties should be assayed in the supernatants, as interpretation of results is greatly simplified with this additional information.

ANALYSIS OF THE REACTION

This discussion will be devoted to an analysis of several mode systems which can serve as a guide for interpretation of the more complex results often observed. For details on background and derivation of the relationships to be discussed, the reader is referred to Kabat and Mayer's book (11).

Precipitation of the antigen-antibody complex is described by 2 different behaviors—the precipitin and the flocculation reaction, represented by the graphs in Figures 3 and 7. The precipitin reaction is characterized by precipitation in the region of excess antibody and a narrow equivalence zone, where neither antigen nor antibody is detectable in the supernatant. The flocculation reaction* is characterized by a zone of complete solubility of the complex in excess antibody and a broad equivalence zone. In both systems the antigen-antibody complex is soluble in excess antigen. Each system will be discussed separately.

Precipitin reaction.—1. SINGLE ANTIGEN-ANTIBODY REACTION.—It is convenient to separate reactions of native globular proteins of up to a molecular weight of 200,000 from reactions of polysaccharides, denatured proteins and proteins of very high molecular weight.

Let us first consider the case of lower molecular weight native globular proteins. We mentioned that the precipitin reaction is characterized by complete precipitation of the antigen in antibody excess. In this region for a single antigen, the total nitrogen precipitated (TN) minus antigen nitrogen added (X) equals antibody nitrogen precipitated (AbN).†

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* It is of singular interest that of all species studied (fish, bird, mammal), the flocculation reaction has been demonstrated only in antiserum from the horse. We recently immunized a donkey by subcutaneous injection of diphtheria toxoid in adjuvants and found that its antiserum gave a precipitin-type reaction. The same held true for human γ_2-globulin, chicken ovalbumin and diphtheria toxoid. Although antibody response was excellent in each case, it was precipitin-type antibody that was found.

When AbN is plotted against antigen added, the curve which describes this reaction is parabolic up to the point where all of the antibody is precipitated. Heidelberger and Kendall (13) have

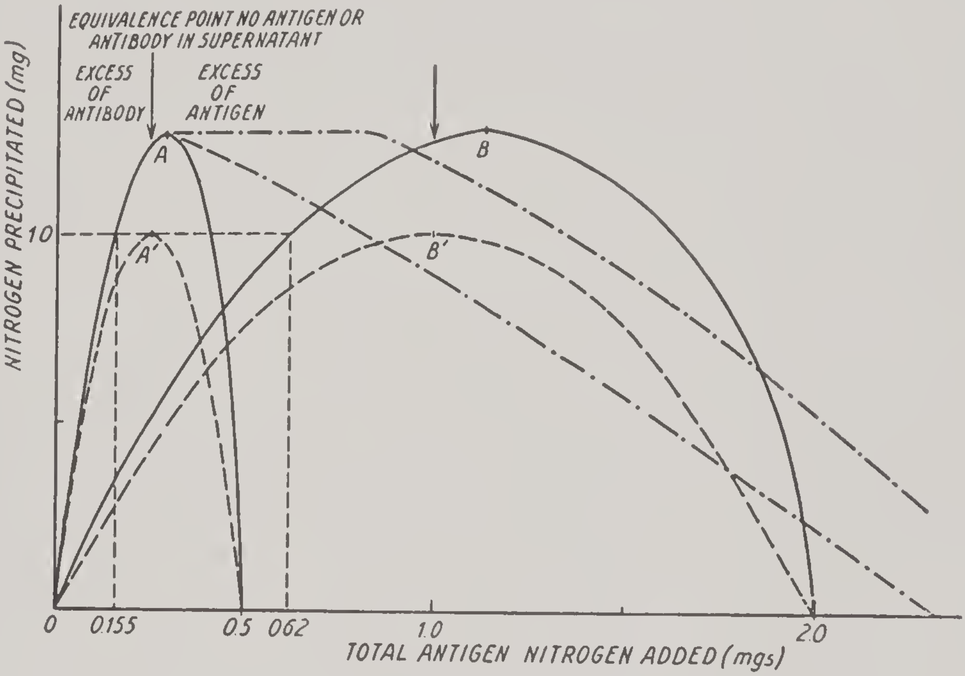

FIG. 3.—Curves of the precipitin reaction. Curve A, described by equation (3) (TN vs. X), represents the reaction of a pure protein. Curve A' is the same reaction described by equation (1) (AbN vs. X). Curves B and B' are the same reaction except that the antigen is 25 per cent pure in the presence of inert protein. Arrows represent equivalence points. Extensions of curve A (—·—·) illustrate behavior of polysaccharides, complex systems, etc. (see text).

derived the following simple relationship which satisfactorily describes the precipitin reaction in the region of excess antibody.

$$\text{AbN} = 2RX - \frac{R^2}{A}X^2 \qquad (1)$$

where AbN = antibody nitrogen precipitated; X = antigen precipitated,‡ in terms of nitrogen for proteins;§ R = ratio $\frac{\text{antibody N}}{\text{antigen N}}$

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† In the case of polysaccharides which contain no nitrogen, TN = AbN; for polysaccharides, antigen added and antibody precipitated are generally expressed in terms of total weight.

‡ For a pure protein, antigen N added equals AbN in the antibody excess zone. Equation (1) would also describe the reaction if X were antigen N added, but the curve would be elongated along the abscissa by an amount depending on the percentage of X in the mixture.

§ For polysaccharides which contain little or no nitrogen, AbN and X are expressed in terms of total weight.

at the equivalence point; A = total antibody N in the unit volume of serum. If we divide through by X, we have

$$r = \frac{\text{antibody N precipitated}}{\text{antigen N precipitated}} = 2R - \frac{R^2}{A}X \qquad (2)$$

The plot of r against X then gives a straight line with slope $-\frac{R^2}{A}$ and intercept at the ordinate of $2R$.

In actual practice, it is TN which is determined. As we mentioned above, up to the equivalence point

$$\text{TN} = \text{AbN} + X$$

and from equation (1)

$$\text{TN} = (2R + 1)\,X - \frac{R^2}{A}X^2 \qquad (3)$$

Let us now consider a hypothetical single antigen-antibody reaction in which the antigen is 100 per cent pure. R and A have arbitrarily been assigned the values of 4.0 and 1.0 mg/N unit antiserum, respectively. Curve A, Figure 3, represents this single antigen-antibody reaction and the equations describing it are

$$\begin{aligned} \text{TN}^{\|} &= 9X - 16X^2 \\ \text{AbN} &= 8X - 16X^2 \\ r &= 8 - 16X \end{aligned}$$

The discussion has, until now, been concerned with behavior of the precipitin reaction up to the equivalence point. We have emphasized the equivalence point for the precipitin system in order to contrast it with the equivalence zone in the flocculation system. The equivalence region in the precipitin reaction is very narrow,[*] and in systems where the antigen is biologically active and can be detected in trace amounts, it can generally be shown that this zone does not extend over more than 5 per cent on either side of the point of maximal antibody precipitated (14). The equivalence zone found in the flocculation system often extends

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[‖] It should be pointed out that the maximum for the curve of TN vs. X is displaced relative to that of AbN; the maximum of the former occurs in the antigen excess region; all antibody is precipitated before this point. The difference between the X maxima necessary to bring equations (1) and (3) to TN maximum depends on the R value. For example, if $R = 1$, the difference between the X maxima of equations (1) and (3) is 33 per cent. If $R = 5$ it is 9 per cent, and for $R = 10$ it is 5 per cent. The increase in TN between the point of disappearance of antibody from the supernatant and the appearance of excess antigen is also a function of R value, and for $R = 1$ the increase is 10 per cent, and for $R = 4$ it is 1 per cent. Since most proteins studied have R values of 4 and larger, this increase in N precipitated is not considered significant. However, the difference in the 2 X maxima is often observed and is more easily detected if the method of testing supernatants is very sensitive (Fig. 6).

[*] Prolonged immunization seems to broaden this considerably (15).

over 100 per cent on either side of the point of optimal flocculation (16).

Analysis of the antigen excess zone is much more difficult. Behavior of the precipitin reaction in this region has not been extensively investigated because (1) equilibrium for precipitation of the complex is hard to attain, (2) no theoretical relationship has been devised to describe it and (3) its quantitative significance is obscure.

Recent investigations (14, 17, 18, 21, 23–25), however, show that this region is extremely useful in the detection of impurities. In general, it may be said that for native globular proteins, the excess of antigen necessary for complete solubilization of the antigen-antibody complex is usually between 2 and 3 times that necessary to reach the equivalence point. For curve *A*, Figure 3, 0.5 mg of added N is required to bring the curve to zero, whereas 0.25 mg is needed at the equivalence point, so that the value here is 2.

One will find systems which approach this description of the precipitin reaction more or less closely. Many known precipitin reactions have been shown to fit the equations up to the equivalence point.† One of the most interesting, described by Adams (19), is the tyrosinase-antityrosinase reaction in rabbit and human sera, in which equation (1) was shown to hold remarkably well even in antigen excess. These observations were extended and confirmed with 2 antigens, diphtheria toxin (14) and β-galactosidase of *Escherichia coli* (21), in which the special nature of the systems allowed preparation of an antiserum which reacted with a single antigen species.‡

High molecular weight polysaccharides (26, 27), artificially diazotized antigens (28), high molecular weight proteins (29, 38), denatured and relatively insoluble proteins (31) exhibit a slightly different behavior. Up to the equivalence point their reactions generally obey the relationships already discussed. However, the antigen-antibody complex is quite insoluble in the antigen excess, and the curves representing the reaction extend out into the antigen excess region in a manner shown by the dotted line continuations of curve *A* Figure 3. The insolubility in antigen excess is probably due to the presence of repeated combining groups on a very large molecule in the first 3 categories of substances mentioned, while in the last 2, insolubility of the antigen itself might be a factor in the insolubility of the antigen-antibody complex in an excess of antigen.

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† The reactions of low molecular weight antigens (8–10,000), such as lysozyme (25), do not seem to fit equation (2). The r values remain constant through the antibody excess region. This means that the curve of AbN vs. X in antibody excess is a straight line instead of a parabola. This reaction is interpreted to mean that lysozyme has a very limited number of antigen groupings or that the antigen-antibody complex is restricted because of steric hindrance.

‡ When the PW8 strain of *C. diphtheriae* is grown in optimal iron concentration, toxins appear in the culture fluid along with a series of atoxic proteins, called P proteins. In higher iron concentration, toxin production is negligible, but the same P proteins appear. Absorption of any antitoxin with P proteins leaves the single antibody (14). The same technique was used to study β-galactosidase, an adaptive enzyme of *E. coli*. Absorption of antisera with extracts of unadapted *E. coli* which contain negligible amounts of β-galactosidase results in an antibody that reacts with a single antigen species (21).

The single antigen-antibody reaction discussed thus far can be obtained by using an antiserum which reacts with a single antigenic species or by using, as precipitinogen, an immunologically pure protein. Let us assume that the antiserum used to give curve *A*, (Fig. 3) reacted with a single antigen species, and as precipitinogen we used a preparation 25 per cent pure. The resulting curve would be identical to curve *A* when the abscissae are correctly reduced. Curve *B* illustrates this point. It can be seen that 4 times as much total antigen must be added to precipitate the same amount of total N in the case of *B* as in *A* therefore preparation *B* contains $^1/_4$ as much of the given antigen as does *A*. This is the principle of the immunologic assay for a given antigenic species. One must be certain that the antiserum does not contain a mixture of unrelated antibodies (discussed more fully later).

2. SIMULTANEOUS REACTION OF 2 ANTIGENS AND ANTIBODIES.—The single antigen-antibody reaction is often unrealized and one is faced with the more complex behaviors due to simultaneous reactions between several antigens and antibodies. We will present as a typical system an imaginary case analogous to some that have actually been found. The analysis depends on the following assumptions: (1) that each reaction takes place independently of the other,§ and (2) that the equation of Heidelberger and Kendall (13) is applicable to each.

Let us consider 2 precipitinogens whose reactions with their respective antibodies can be represented by the equations already discussed. Equation (3) (TN) will be used as a 1st approximation and it will be assumed that the equation applies in antigen excess. Consider now 2 antigens and antibodies (*1* and *2*) with the following constants

1

$R_1 = 10$
$A_1 \text{ max} = 1.0$
$\text{AbN}_1 = 20\,X_1 - 100\,X_1^2$
$\text{TN}_1 = 21\,X_1 - 100\,X_1^2$

2

$R_2 = 5$
$A_2 \text{ max} = 0.1$
$\text{AbN}_2 = 10\,X_2 - 250\,X_2^2$
$\text{TN}_2 = 11\,X_2 - 250\,X_2^2$

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§ Simultaneous injection into an animal of 2 different antigens results in formation of 2 discrete antibodies, never a single antibody effective against both antigens (32-34). Thus each precipitin reaction should take place independently of the other. If the mixture of precipitinogens contains cross-reacting structures, the problem is more complicated and often more interesting. This will be discussed in detail later.

Suppose that a given protein preparation contained 90 per cent X_1 and 10 per cent X_2. Figure 4 represents the reactions of this system. At 0.11 mg of added antigen N, component *1* is at its equivalence point, whereas there is 0.01 mg antibody N to component *2* still unprecipitated in the supernatant. Tests of the supernatant at this point will show antibody excess. A further addition of total antigen will result in an antigen excess of component *1* and antibody excess to component *2*. Thus the clearest

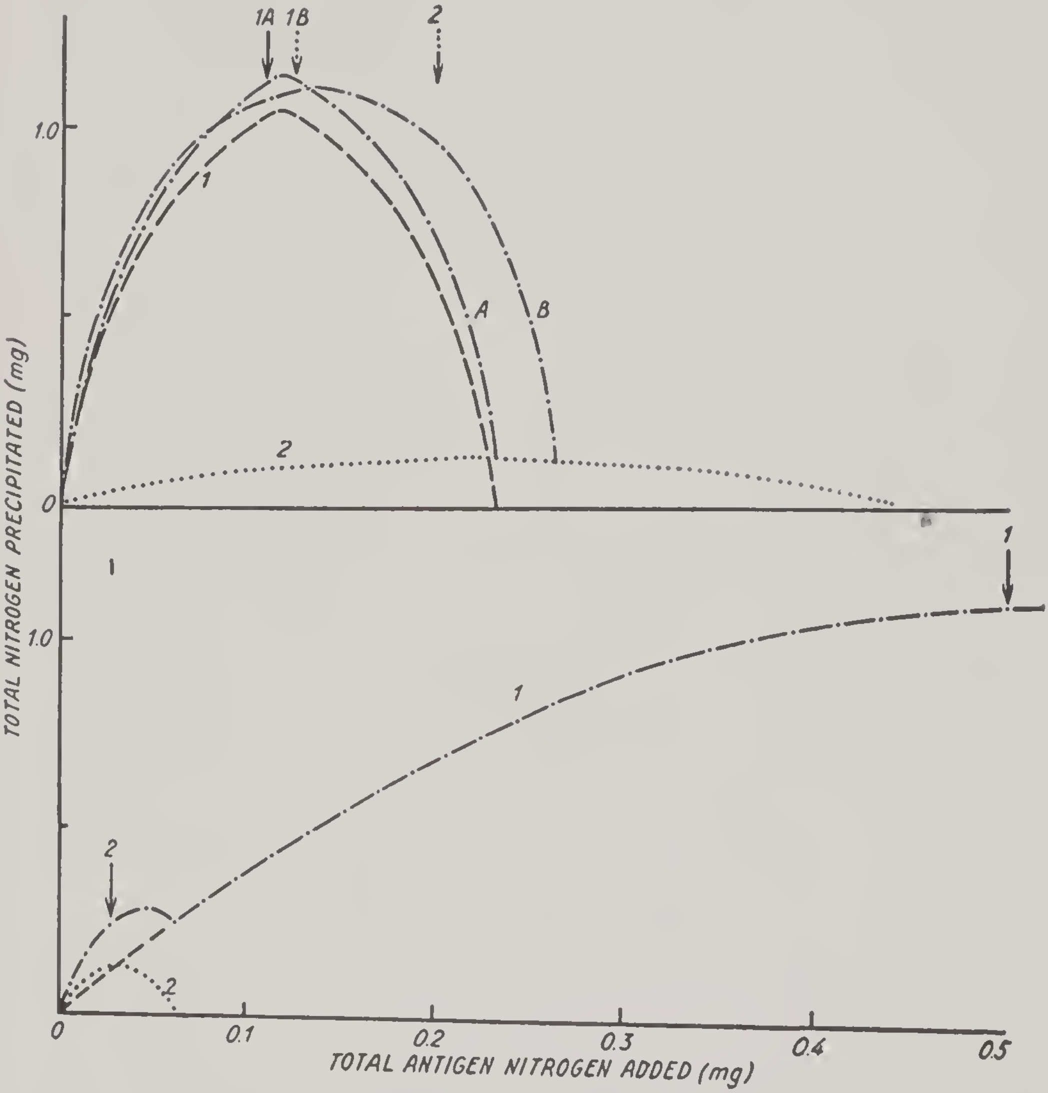

FIG. 4.—*Above:* Reaction of a purified protein preparation containing 2 components, *1* and *2*. Curves *1* and *2* represent reactions of components *1* and *2* as though they were alone. Curve *A* is the resultant sum of curves *1* and *2* and represents the reaction actually determined. Curve *B* is the resultant sum when concentration of component *2* is slightly changed (see text). *1A* indicates equivalence point of component *1* in reaction of mixture that is represented by curve *A*, and similarly for *1B*. Equivalence point of component *2* is represented by *2* with arrow. *Below:* Curves of reaction of components *1* and *2*, entirely different proportions, with the same antiserum (see text).

evidence for more than 1 reacting substance is the presence of both antigen and antibody in any given supernatant.[‖] We owe the precise statements of this condition for immunologic homogeneity to Kendall (35), Culbertson (36) and Weil (37).

Presence of an equivalence point would be a sufficient criterion of immunologic purity if the precipitin reaction as a test for antigen and antibody excess were sensitive enough. In fact, in the rather obvious example given here, one might have missed the zone of simultaneous excess of both antibody and antigen because the test for excess of antibody by the precipitin reaction will detect at best about 3–5 μg N in 2–5 ml. If component *1* were 80, not 90, per cent of the total and component *2* were 10 per cent, the rest being inert, the zone of excess antigen and antibody would not have been detected (see Fig. 4). At the equivalence point of component *1*, only 1 μg antibody N against component *2* would remain, and we would say that the system of 2 components had an equivalence zone. This being our only criterion, the 2 component reactions would be called a single one. Now let us look at the antigen excess zone. The summation curve drops sharply, then levels off and trails out into far antigen excess. This does not in itself mean that the system is inhomogeneous, but it acts as a warning to look further. Examples of this type of complex curve were given by certain preparations of diphtheria toxin (14), ovalbumin and conalbumin (17), β-lactoglobulin (23), γ-globulin (18), β-galactosidase (21).

To test the system, another mixture of antigens is used as precipitinogen, e.g., the starting material from which component *1* was purified. For demonstration, suppose that the starting system consisted of 20 per cent component *1*, 70 per cent component *2* and 10 per cent inert material (see Fig. 4). At 0.028 μg antigen N added, component *2* is at its equivalence point. Between this point and 0.50 μg antigen N added, excess of both antigen and antibody would be easily detectable in the supernatant, whereas with the purified antigen it would not be observed. Examples of investigations using this technique are those on conalbumin (17) and β-lactoglobulin (23). Scherp (40) devised a method for resolving the reaction due to each component in that mixture and for determining the antibody nitrogen to each component.

[‖] It is well to point out that the tests of supernatants would also show both antigen and antibody excess if (1) the antibody antigen complex were highly soluble (38), or (2) in the presence of degraded (39) or cross-reacting antigens.

Dr. Oudin discusses the agar diffusion technique in the chapter which follows. Therefore this method, which has become an important arm of the immunochemist, will only be mentioned here. However, it is important to check both antigens and antisera for homogeneity by this method. In addition, supernatants can also be tested by this method, thus effecting a considerable increase in the reliability of these tests.

In summary, then, the testing of a given purified protein antigen A should be done in the following manner.

1) Anti-*A* sera should be reacted with (*a*) antigen *A* itself, (*b*) the crude starting mixture from which *A* was isolated and (*c*) fractions which contain higher concentrations of probable impurities.

2) Antigen *A* should be reacted with antisera prepared against materials likely to contain impurities in *A*, such as the crude starting material or fractions of it. For example, anti-whole egg white sera, anti-whole serum sera, anti-milk whey sera and anti-allantoic fluid have been used to test purified ovalbumin (17), γ-globulin (18), β-lactoglobulin (23) and encephalomyelitis virus (41), respectively.

3) In cases of doubt due to very weak reactions, the antisera can be concentrated by fractionation and restudied (for an example see (17)). A single antigen-antibody reaction will show an equivalence zone, and in the case of native globular proteins of molecular weights up to about 200,000 the antigen-antibody complex will be readily soluble in excess antigen. The reverse, i.e., a curve which drops sharply in antigen excess, is not by itself evidence of a single antigen-antibody reaction, nor is the trailing out of the curve into far antigen excess necessarily evidence of reactions of impurities. All of the previously discussed factors must be considered. The antigen excess region of the curve should be used only as a guide. All of the curves obtained under condition (1) should be superimposable over the entire region of precipitation.

If an antiserum is to be used to assay for a given antigen in a complex mixture, it must be demonstrated either that the antibody preparation will react with only a single antigenic species or that the reactions of impurities and antibodies to them are not significant in the range of values studied. It is quite possible that antibody directed against a trace of impurity, too small to detect in the purified antigen, would become very significant in the analysis of crude mixtures containing a greater concentration of impurity. It is therefore very important that condition (1) be studied. The problem was analyzed in detail by Cohn *et al.* (18). In addition, cross-reacting substances render the use of antiserum unreliable for assay of a given antigen. An antiserum determines the total of antigenically similar material which is the sum of the antigen under consideration and the cross-reacting substances. Therefore a serum standardized with a purified single antigen would give high values for the concentration of this antigen in the crude mixture containing a cross-reacting antigen.

Furthermore, one should be very careful to make certain before using an antiserum analytically that the antigen studied from

1 source is the same as that assayed for in another source. For example, anti-serum albumin serum has been used to study the synthesis of this protein by liver slices without first demonstrating that the liver protein and serum protein were identical (6). It is possible that the liver protein is a cross-reacting substance, in which case interpretation of the results would be quite different.

As a final point, certain differences between molecules are not distinguishable immunologically. For example, deamination (20) or treatment with formaldehyde under controlled conditions (77) results in proteins immunologically indentical with the untreated substance.

3. REACTIONS OF SUBSTANCES WITH ACTIVITY.—If the antigen under consideration has a known measurable activity, e.g., is an enzyme, toxin, virus, or hormone or is characteristically marked, i.e., colored, radioactive or with special groupings, the immunologic problem becomes greatly simplified. The antigen-antibody reaction can be analyzed independently of reactions of antibodies to unrelated substances by use of the 2 markers on the molecules, specific activity* and antigenicity. In addition to testing the supernatants for excess antibody or antigen by the presence or absence of a precipitate, the supernatants are examined for excess of specific activity. If, for example, the specific activity was detected together with an excess of precipitating antibody in any given supernatant or if an excess of precipitating antigen were present in a supernatant with the anti-specific activity, the reaction of an impurity would be revealed.

Let us consider now the question of identification of specific activities with given protein molecules. Suppose that we isolate an enzyme preparation *A* which is capable of catalyzing reactions *Y* and *Z*. On what basis can we decide whether 1 enzyme is catalyzing these 2 reactions or whether 2 separate enzymes are present? The immunologic method is one of the most sensitive. Antisera are prepared to preparation *A*. If antibody directed only against *Y* or only against *Z* is produced in any serum, then *Y* and *Z* are associated with different antigenic species. However, in most cases, antibodies will be directed against both. The immunologic analysis of this system does not involve the precipitates. The supernatants are analyzed for anti-*Y* and anti-*Z* and for *Y* and *Z* activities. Two results are possible: either (*a*) the equivalence points of *Y* and *Z* are *not* identical and the separation of *Y* and *Z* is effected immunochemically, or (*b*) the equivalence points for antigen *Y* and antibody *Y* and antigen *Z* and antibody *Z* are identical and the ratio of $\frac{\text{activity } Y}{\text{activity } Z}$ remains constant throughout the antigen excess zone.

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* Any distinctive property of the preparation could be useful, e.g., absorption due to nucleic acid in a nucleoprotein (22).

Considering possibility (*a*) first, we could say that activities *Y* and *Z* are associated with antigenically distinguishable species, although we could not decide by this test alone whether these "distinguishable species" cross-react. Rigorous resolution of this problem would require separation of the 2 or the use of reactions in gels, which will be discussed. If possibility (*b*) were true, we could say that *Y* and *Z* are associated with identical antigenic species. However, this does not mean that the same specific grouping is involved in both reactions. One need only recall that tetanus and diphtheria antitoxins are indistinguishable not only as antigens but by all physical criteria, yet they are separable by specific precipitation with their respective antigens. An analogous situation in other protein systems has not been found, but studies of this type are not extensive enough. There are interesting examples of use of this technique to show that ferritin and the vasodepressor substance (42), the α-toxin and lecithinase of clostridia (43), the lactase and β-galactosidase of *E. coli* (21), and the polysaccharidases acting on S VIII and S III pneumococcus polysaccharides (44), respectively, are associated with the same antigenic species. There are numerous examples in the literature of 2 activities supposedly due to a single molecular species; they could be tested rigorously by this technique, i.e., ricin and protease (45), malic and oxaloacetic decarboxylases (48), maltase and α-phenylglucosidase (47), oxytoxin and vasodepressor hormones (48), and catecholase and cresolase (49).

This type of analysis is applicable to the testing of any specific property of the molecule. For example, suppose that enzyme preparation *A* contains activity *Q*, copper and iron and that we wish to determine whether this activity is associated with an iron or copper protein or neither. Analysis of supernates by the method already described would give an equivalence zone for activity *Q* and either copper or iron, or neither at the same point. If, for example, the copper/activity *Q* ratios were constant over the entire range, whereas the iron/activity *Q* ratio changed, we would say that we had a copper enzyme protein. This analysis can, under certain conditions, become extremely complex, but either by a proper choice of serum or by slight fractionation, complexities due to the presence of several unrelated iron or copper proteins or copper and iron enzymes with cross-reacting protein moieties could be distinguished.

This method of analysis depends on the use of a specific combining reagent, antibody, to resolve 2 different activities into distinct physical phases by means of precipitation. The foregoing reasoning is applicable in a slightly different manner to systems in which this phase separation does not occur. Suppose that we used the neutralization reaction, e.g., inhibition of activity without precipitation. Two situations may be found. In the first case, activity *Y* is inhibited by a given antiserum but activity *Z* is not. No conclusion can be drawn as to whether the combining groups responsible for both activities are identical unless it is known that activity *Z* can be inhibited by an antibody directed against it. This latter condition can only be satisfied when both activities *Y* and *Z* are inhibited by an antiserum preparation. Here, if activity *Z* and activity *Y* are associated with the same combining groups, at the neutralization point of a fixed unit of activity *Z*, one would expect to observe the same residual unit of activity *Y* with many different preparations of antisera. Any other result indicates that the 2 activities are associated with different combining groups, but not necessarily different protein molecules. This problem can become technically very complicated with certain antigens which show complex behavior in the neutralization reaction, e.g., phage.

One of the best examples of theuse of these methods is the analysis by Cohen and Argobast (50) for the purity of various *E. coli* B phage preparations. They found that antisera to the uninfected host *E. coli* would not precipitate or inactivate the virus activity but would remove the non-desoxyribonucleic acid phosphorus from the virus preparations. Phage that was further purified after treatment with these antisera had all of its phosphorus as desoxyribonucleic acid phosphorus. The specific precipitates contained the ribose nucleic acid, and the authors correctly concluded that it was not a part of the virus but an unrelated impurity in the purified preparations.

Although we have been using as examples the reactions of proteins, the same principles apply to polysaccharides. Kabat and his co-workers have developed this type of analysis extensively. One example is of great interest. As we have observed, all of the antigen is precipitated in presence of excess antibody, so that, for a system involving a single antibody, the ratio of the antigen in the specific precipitate to the total preparation added gives the degree of purity directly. Certain preparations of group *A* substance contained 33 per cent glucosamine. Anti-*A* sera precipitated only about 56 per cent of the glucosamine. The inactive material which was not precipitated by anti-*A* sera was identified as group *O* substance. Of course, impurities not containing glucosamine would not be detected by this method (51). Another example is to be found in the work of Perlman and Goebel (52).

4. IMMUNOLOGIC METHODS IN STUDY OF PROTEIN BIOSYNTHESIS.—The identification of cross-reacting proteins in a given preparation is important in the study of biosynthesis of proteins. On the basis of numerous studies (53), we know that the phenomenon of cross-reaction is due to *structurally related substances.* The presence in a given cell of 2 proteins which cross-react, and are therefore structurally similar, implies an ontogenic relationship between them; e.g., 1 might be a precursor of the other, or they might have a common origin. Structurally related proteins probably would behave similarly in fractionation by any technique—solubility, electrophoresis or ultracentrifugation—and therefore would be the most likely "impurities." Cross-reacting antigens in a given protein preparation have been identified, e.g., ricin and hemagglutinin (54), high and low molecular weight γ-globulins (55). We will here describe another instance as an example of a type of reaction which can be expected (56, 57).

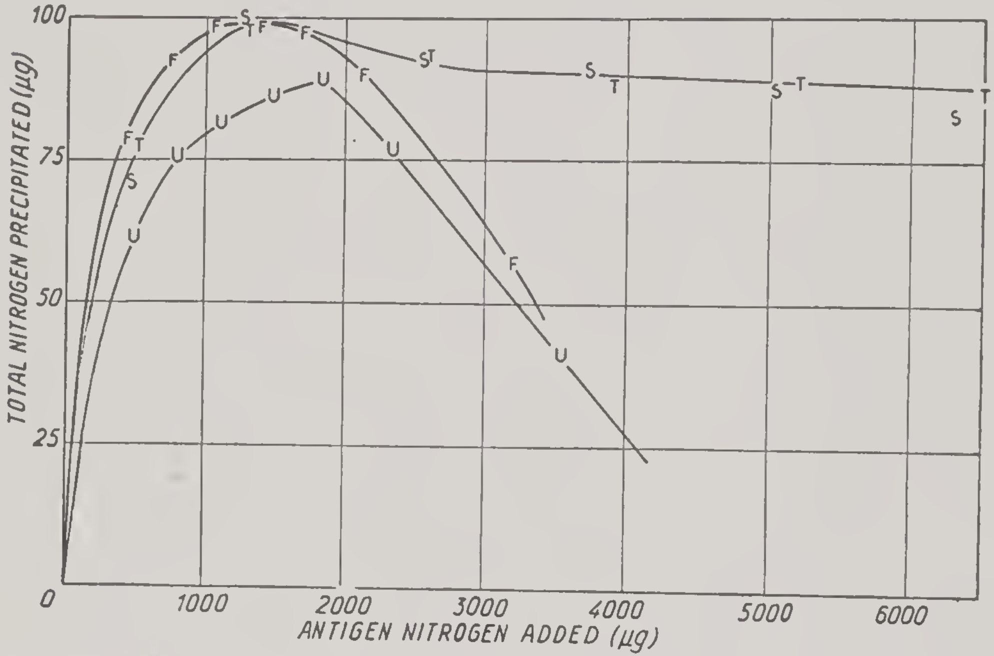

FIG. 5.—Reaction of β-galactosidase (*Gz*) and structurally related proteins with rabbit anti-*Gz* serum. Curve *U*, reaction of extracts containing *Pz* from unadapted cells. Curves *F* and *T*, reactions of extracts of cells adapted to different extents which contain both *Pz* and *Gz*. Curve *F* represents reaction of the extract containing the greater proportion of *Gz* relative to *Pz*. Curve *S*, reaction of special mixtures of extracts *F* and *U* such that relative proportion of *Pz* to *Gz* is the same as extract *T*. For curves *S* and *T*, equivalence point T is close to 1,100 µg N added and equivalence point G is at 6,300 µg N added. For curve *F*, equivalence point T is at 1,000 µg N added and equivalence point G is at 1,600 µg N added.

The β-galactosidase (*Gz*) is an adaptive enzyme of the ML strain of *E. coli* and it is induced by a series of substances having the unchanged galactosidic structure. Organisms grown on noninducing sugars possess only traces of *Gz* but large amounts of a protein (*Pz*) which cross-reacts with anti-*Gz* sera. *Pz* is also present in adapted cells and, in fact, contaminates the most highly purified preparations of *Gz*. Rabbit antisera to *Gz* precipitate but do not inhibit enzymatic activity. *Pz* precipitates about 80–90 per cent of the anti-*Gz*, and the antisera absorbed with *Pz* give the single antigen-antibody reaction described previously.

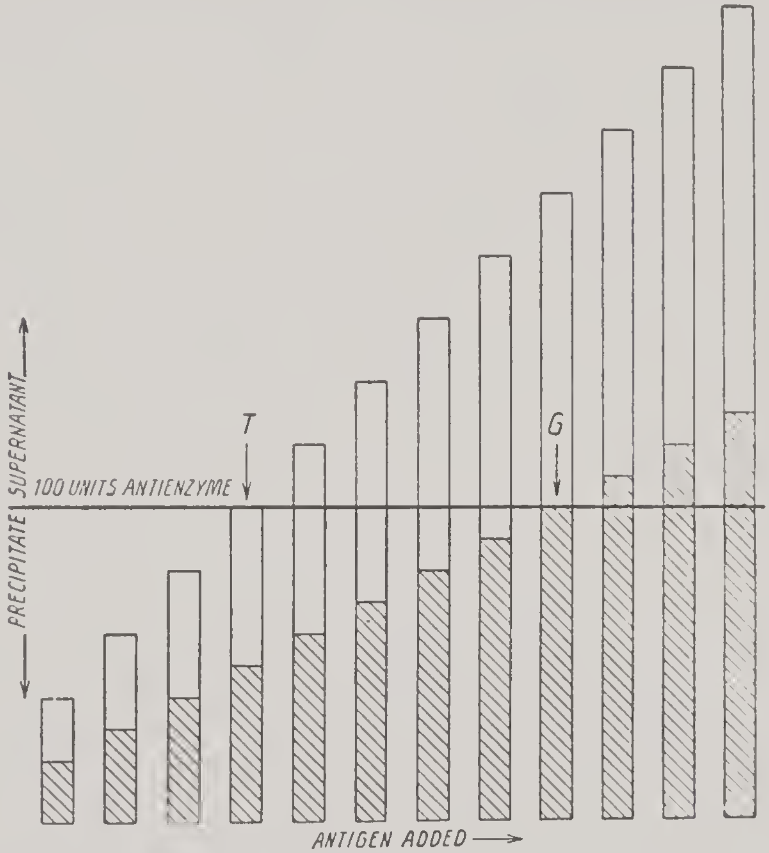

Fig. 6.—Diagrammatic representation of reactions shown in Figure 5. Striated lower bar represents *Gz*. Clear upper bar represents *Pz*. Horizontal line represents a fixed quantity of antiserum defined as 100 units (see text for explanation). Zone 1, bars 1–3; zone 2, bar 4; zone 3, bars 5–8; zone 4, bar 9; zone 5, bars 10–12.

The interesting property of this system for us is the behavior of mixtures of the 2 precipitinogens *Gz* and *Pz*. Figure 5 describes the quantitative curves of the system, and Figure 6 the diagrammatic representation. The equivalence point *G* of the *Gz* and anti-*Gz* reactions can be expressed in terms of *units of enzymatic activity precipitable by a given number of units of antibody.* This equivalence point is independent of the presence or absence of *Pz*. In other words, *Gz* has a higher affinity for the anti-*Gz* than has

Pz, and in mixtures of the 2, *Gz* precipitates preferentially. In fact, *Gz* will displace *Pz* from the soluble complexes in the antigen excess zone. The behavior of this system is entirely analogous to the cross-reaction in horse antisera of S VIII and S III pneumococcus polysaccharides (58). As one adds increasing amounts of a mixture of *Pz* and *Gz* to a fixed, given quantity of antiserum, several regions can be defined (Fig. 6). These zones can be described as follows, as we move in the direction toward excess antigen.

1) All of *Pz* and *Gz*, as well as part of the antibody, precipitates. There is excess antibody in the supernatant.

2) All of *Pz*, *Gz* and antibody precipitates. This is called *equivalence point T* and defines the total antigenically similar material (*Tz*). *Tz* equals the sum of *Gz* and *Pz*.

3) All of the *Gz* and antibody precipitates, along with part of *Pz*. Excess *Pz* appears in the supernatant. *Gz* precipitates preferentially, and the unreacted combining groups of the antibody take with them part of *Pz*. Notice that TN in this region is constant.

4) All of *Gz* and antibody is precipitated. All of *Pz* remains in the supernatant and is quantitatively separated from *Gz*. This is *equivalence point G* and defines the quantity of enzymes (*Gz*) as an antigen.

5) Further additions of antigen result in an excess of *Gz* in the form of soluble complexes, and all of *Pz* is in solution.

One can determine the concentration of *Pz* relative to *Gz* in the following manner. A convenient volume of antiserum is arbitrarily defined as equivalent to 100 units of antibody. One hundred units of *Gz* will bring the system to equivalence point G; 100 units of total antigen will titrate to equivalence point T. The difference between the combining units *Tz*/unit vol and combining units *Gz*/unit vol is the combining units *Pz*/unit vol. It should be stressed that this titration using 2 specific markers of the molecule is independent of unrelated antigen-antibody reactions. Depending on the ratio *Pz*/*Gz*, TN remains constant over a variable range, which is wider as the ratio is higher. If we did not have the specific enzymatic activity of *Gz* to guide us, we might conclude on the basis of a test of supernatants that we had a single antigen-antibody reaction, because at equivalence point T no antigen or antibody would be detectable in the supernatants. Here again, the antigen excess zone proves important.. The antigen-antibody complex appears to be insoluble in excess antigen. The reaction resembles that due to a polysaccharide. If neither cross-reacting antigen has a measurable specific property, the only guide to analysis is the form of the curve in antigen excess. Any supernatant which corresponds to the flat region of the curve in the antigen excess zone is used as a precipitinogen, because a separation of the 2 cross-reactants might be effected here. The new reaction, in contrast to the old one, would not show this flat region, and the new precipitinogen should behave like a cross-reacting substance, precipitating less antibody/unit antigen than the homologous antigen. On the other hand, if the 2 cross-reacting proteins had identical affinity, it would be impossible, in the absence of another specific marker, to distinguish them by these immunochemical methods alone. The use of reactions in gels might resolve them.

The behavior of *Pz* and *Gz* suggests that a similar system of cross-reacting proteins is present in preparations which show the same type of quantitative precipitin curves. Here we can only speculate. Jager *et al.* (59) and Kabat *et al.* (60) have suggested that γ_2-globulin components might cross-react with other serum proteins. Cohn, Deutsch and Wetter (18) have suggested that their results do not rule out the possibility of the reactions of unrelated components common to various γ-globulin preparations. However, the form of several of the curves published by the latter workers supports the suggestions of Jager, Kabat and co-workers. For example, the reaction of γ_{II}-globulin with anti-γ_2-globulin sera could be due to the reaction of 2 cross-reacting proteins, for the curves show that the precipitinogen is remarkably insoluble in excess of antigen, quite unlike ordinary protein-antiprotein reac-

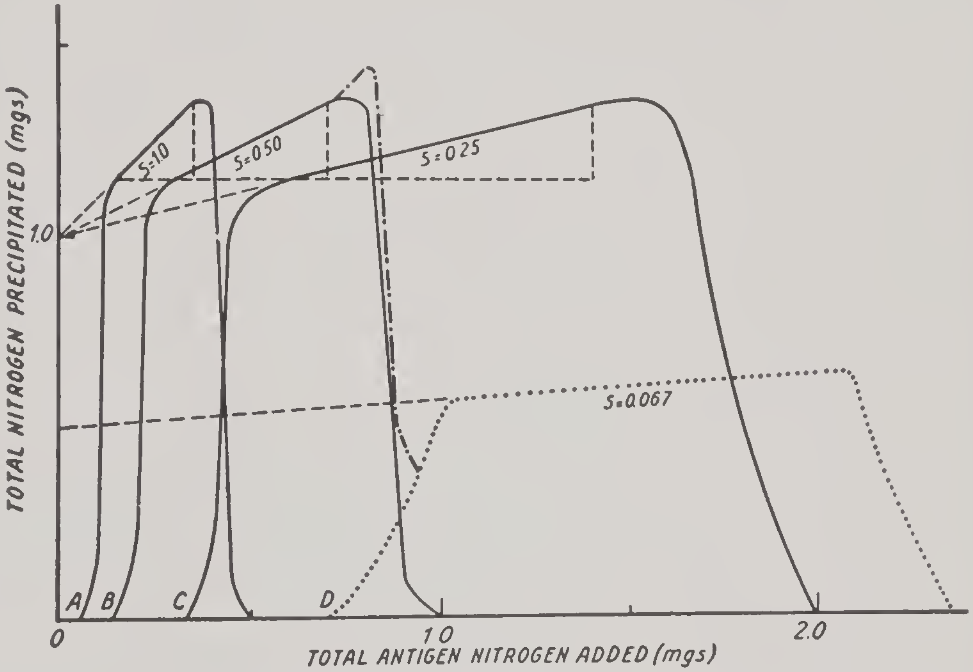

FIG. 7.—Flocculation reaction (see text). Overlapping portion of curves *B* and *D* is represented by the line —·—·—· *S* = slope.

tions. This was not explained. Similar findings have been reported for reactions between chicken whole egg white and anti-conalbumin sera (17) and milk whey proteins and anti-β-lactoglobulin sera (23). These interesting systems deserve further investigation.

Flocculation reaction.—This antigen-antibody reaction, first interpreted in quantitative terms by Pappenheimer and Robinson (16) and represented by the curve in Figure 7 is relatively simple to analyze. It is characterized by precipitation in a narrow region and a broad equivalence zone. Curve *A* represents the reaction between a pure antigen and its flocculating antibody. Since the total antibody is a constant and all antigen and antibody are precipitated in the equivalence zone, a straight line represented by equation (4) is obtained for this region.

$$\mathrm{TN} = PX + A \tag{4}$$

where X and A are defined as in equations (1), (2) and (3) and P is the ratio of specifically precipitable antigen N to total N added, in other words, the percentage purity in terms of nitrogen.

Curve *A* of Figure 7 has been drawn to permit comparison with curve *A* of Figure 3. Total antibody is chosen as 1.0 mg N and $R = 4$ at the point of optimal flocculation. A 100 per cent pure protein was used as precipitinogen and the slope of the line is therefore 1.0. Curves *B* and *C* show what happens when precipitinogens, 50 and 25 per cent pure in terms of N, are treated with this antibody; the slopes of the lines in the equivalence zone are 0.50 and 0.25, respectively. All of these lines extrapolate to 1.0 mg N, which is the total antibody.

To assay for a given antigen in a mixture, it is enough to determine TN at any point in the equivalence zone. Since X and A are known, equation (4) can be solved for P, which is the percentage purity. This technique has been used to determine the purity of diphtheria toxoids (14).

A curve of the type shown in Figure 7 provides clear evidence that a single antigen-antibody reaction is involved. This means simply that antibody reacting in the system precipitates a single antigenic species. The flocculating system cannot be used to detect small amounts of impurities, as the determination of the slope of the line is only good to about 2 per cent, so that given protein preparations between 96 and 100 per cent pure will be indistinguishable.† However, the flocculating system, in the equivalence region, separates the precipitinogen under investigation from its impurities, which remain in the supernatants. Rabbit antisera prepared against this same antigen might contain antibodies against impurities, which could be detected and in some cases even assayed for in the supernatants in the equivalence zone of the flocculation reaction. This was actually done to detect impurities in certain conalbumin preparations (17) which gave a curve of the flocculation reaction with a slope of 1 in the equivalence zone. The latter technique is extraordinarily sensitive, and one can detect as little as 0.1 per cent impurity with no difficulty.

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† This, of course, does not apply if the flocculating antibody is directed against a substance considered as impurity.

Another extremely useful property of the flocculation reaction is that precipitation takes place in zones. This makes it possible (1) to observe the reactions of minor components which may precipitate in other zones, and (2) to separate these reactions with a minimum of difficulty.

An example is given in Figure 7. Our hypothetical flocculating antiserum showed 2 zones of optimal flocculation with a given antigen preparation. The curves obtained on quantitative analysis ran into each other, as shown by curves *B* and *D*. The straight line portion of curve *B* was sufficiently distinguishable to show a slope of 0.50 and that of the 2d curve *D* was 0.067. This means that precipitinogen *B* is present in 50 per cent and *D* in 6.7 per cent of the total preparation, and extrapolation of these lines to zero shows that 1.0 mg antibody N is directed against precipitinogen *B* and 0.50 mg against *D*. A rather crude separation such that precipitinogen *B* is purified to 60 per cent and *D* reduced to 3 per cent would completely separate these systems immunologically. It is obvious that even if the 2 curves showed 90 per cent overlap, the complex reaction could be observed and simple techniques that would change the relative concentrations of the precipitinogens by as much as 10–20 per cent might separate the curves sufficiently for adequate analysis. Experimentally, the best examples of this type behavior are the reactions of chicken ovalbumin and conalbumin with horse antisera (17) and the double zones of flocculation found with antitoxin to scarlet fever toxin (61). It is obvious that one can determine the concentration of a protein which has not yet been isolated if a flocculating antibody to it is available. This was done successfully for diphtheria (16), tetanus (62) and scarlet fever toxins (63).

Despite the great usefulness of this system,‡ it is rarely used, for several reasons. (1) The system has only been observed in sera from horses, whose care makes laboratory use difficult. (2) Often the period of immunization must be long, and large quantities of antigen are required. (3) It is not always possible to obtain flocculating antisera which give clear-cut results.

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‡ One other piece of information that may sometimes be obtained from analysis of the specific precipitate at the point of optimal flocculation is worth mentioning. If one assumes that the molecular formula of the specific precipitate is 2 molecules of antibody to 1 molecule of antigen, the molecular weight of the antigen can be calculated. The molecular weight of horse pseudoglobulin is 160,000. This calculation was successfully applied to diphtheria toxin (73) and ovalbumin (65). One other case recently analyzed is of interest. The flocculation reaction between conalbumin and its antibody led to a calculation of the molecular weight as 73,000–78,000. At

It is unfortunate, nevertheless, that more work has not been done on elucidating the factors which differentiate precipitin from the flocculation-type antibody. Convenient production of the latter type would be of enormous value to immunology. Flocculating antibodies have been described for a large series of proteins, diphtheria (16), tetanus (62), scarlet fever (63) and botulinus toxins (64, 30), chicken ovalbumin (65) and conalbumin (17), human and rabbit serum albumins (66, 67) and hemocyanin (68).

To obtain the typical curves of the flocculation reaction, high titer antisera must be used. Even in the reaction of diphtheria toxin, which has received most extensive study, weak antisera give apparent precipitin-type curves. Other aberrations are found when nonspecific precipitation (69) or combined reactions of flocculation and precipitin types occur (70, 71). In addition, there is preliminary evidence that certain protein antigens, e.g., γ-globulin, induce precipitin antibody in the horse (72). One cannot expect that every horse immunized with a protein will give antiserum which will show a typical flocculation reaction.

In addition, it should be mentioned that horse antisera to polysaccharide give typical precipitin-type reactions which might be due to the peculiar characteristics of the polysaccharides (large linear molecules with repeated combining groups) or to the special properties of polysaccharide antibodies which are of much higher molecular weight and greater insolubility than the flocculating antibodies. With respect to this question, the interesting observation of Grabar and Staub (74) should be mentioned. Horse antisera against the glycoprotein of the anthrax bacillus were shown to give a precipitin-type reaction with this precipitinogen. However, the pseudoglobulin fraction reacted to give a flocculation reaction. It seems as though this polysaccharide-protein induced the formation of both types of antibody which were separable with euglobulin and pseudoglobulin fractions.

Pappenheimer introduced the interesting hypothesis that the flocculating antibody differed from the precipitin antibody in

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the time of this calculation the molecular weight of a purified preparation as determined from sedimentation-diffusion data was found to be 87,000. The immunologic value appeared low. Two years after this work, Warner and Weber (78) crystallized conalbumin, and from its iron content and iron-binding capacity reported the molecular weight to be 70,200–76,600, a value in striking agreement with the immunologic one.

that the former had both combining groups on the same side of the molecule and that 1 site interfered with the reaction of the other (73). Kendall has postulated the presence of 2 combining groups with different reactivity and derived a mathematical relationship which describes the flocculation reaction remarkably well (75). There are other possible explanations which can be tested.

The flocculating system might be due to a mixture of precipitating and nonprecipitating antibodies. The latter inhibit precipitation in large antibody excess and in the equivalence zone behave much as do the univalent antibodies in the cross-reaction between pneumococcus polysaccharide type III and anti-type VIII sera (76); i.e., the linear portion of the curve of a flocculation would result from combined complete precipitation of precipitating and nonprecipitating antibodies. Evidence in support of this theory is that horse antisera have large amounts of nonprecipitating antibody in the early stages of immunization, which means that the animal does synthesize this type of antibody. This hypothesis predicts that correct artificial mixtures of precipitating and nonprecipitating antibodies would give a typical flocculation reaction with the corresponding precipitinogen.

ADDENDUM

The reviewers have felt that the author oversimplified the discussion of the flocculation reaction, for recently 2 papers (69, 79) have appeared in which certain quantitative aspects of the flocculation reaction as described here have not been confirmed. The reviewers have considered these papers of sufficient importance to merit an extended discussion and have suggested that they be analyzed in detail.

All workers agree that the *flocculation reaction* is characterized by solubility of the antigen-antibody complex in both antigen and antibody excess; therefore the reaction takes place in a zone. In fact, many horse antitetanus and scarlet fever antitoxins react in more than 1 zone, and the problem is to find which zone is due to the toxin and which to unrelated material, a question only resolved by analyzing the supernatants for disappearance of specific activity (61). Precipitation in a zone has also been confirmed by investigations on the reaction in gels (see Oudin, this volume).

The discussion which follows involves the "straight line" portion of the curve from which the percentage purity of a given antigen can be determined. Since this question has been raised most pointedly by Pope *et al.* (79) with regard to the diphtheria toxin-antitoxin reaction, this system and their recent report on it will be analyzed. These workers find that the reaction takes place in a zone but state that they "have failed to obtain a single curve of the type reported by Pappenheimer and Robinson (1937) or Cohn and Pappenheimer (1949) for horse antitoxin." This refers to the "straight line portion" of the curve.

It has been known since the time of Ehrlich and confirmed by many workers that the reaction between diphtheria toxin and *horse* antitoxin exhibits an exceptional behavior, in that the difference between the L+ dose and the Lo[*] dose of toxin is *not equal* to 1 minimum lethal dose but is much greater (about 30 times) and that in general the L+ and Lo doses are greater than the Lf dose determined at the point of optimal flocculation. The uninitiated reader need not fatigue himself over these units, for the argument should clarify itself without them. In terms of quantitative immunochemistry the discrepancy between L+ and Lo, taken together with the large Danysz effect observed after all of the antitoxin is combined, signifies *that the reaction between diphtheria toxin and horse antitoxin is characterized by a broad equivalence zone.*[†]

With some hindsight, then, one can state that it should not have been a surprise that Pappenheimer and Robinson found a linear relationship between toxin N added and total N precipitated, since it was implicit in the already known finding that with horse antitoxin there is a large series of antigen-antibody mixtures over which all toxin and antitoxin is precipitated. In fact, had Pappenheimer and Robinson uncovered any other relationship the explanation of the relationship between the various units (L+, Lo, Lf) of toxins and antitoxins would have remained obscure. In 1937, Pappenheimer and Robinson reported that this finding was reproducible in 6 different sera, and since then many laboratories have confirmed their observations. Furthermore, similar results have been obtained with at least 6 other antigens, as mentioned in the text.

It might be pertinent to point out that it would not have been necessary to repeat their curve with many sera. It suffices to have *one* system which follows the "ideal" flocculation reaction to draw precise conclusions concerning the percentage purity of various toxin preparations. The fact that one can find systems, as have Pope *et al.*, which for one reason or another do not show the "ideal" reaction is of little significance from the point of view of determining homogeneity of proteins. The recent reports of Van Heyningen *et al.* (69) and Pope *et al.* (79) do not deny the essential character of the flocculation reaction. They demonstrate, rather, that it can be complicated by unrelated factors. It is obvious, therefore, that if the straight line region were present, it could easily be masked by nonspecific precipitation (69) or by overlapping precipitation of unrelated systems (79). But the danger here is no greater nor is it subject to different controls than in the precipitin system: with due regard to similar complications, rabbit antisera have been used to assay many systems for given antigens. Since Pope *et al.* (79) do not report on analyses of the supernatants for the reactions they studied, it is impossible to find out over what region of their curves the straight line portion (equivalence zone) could be expected and thereby calculate how much is contributed by unrelated systems.

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[*] *The Lo dose* of diphtheria toxin is the largest amount of toxin that, when mixed with 1 unit of antitoxin and injected subcutaneously into a guinea pig of 250 g weight, will, on the average, give rise to no observed reaction (80).

The L_+ dose of diphtheria toxin is the smallest amount of toxin that when mixed with one unit of antitoxin and injected subcutaneously into a guinea pig of 250 g weight, will, on the average, kill that guinea pig within 96 hr (80).

[†] Analysis of the phenomenon has been given in detail elsewhere (14), and it has been pointed out that units at present in use depend on the broad equivalence zone of the flocculation reaction and would not apply to the precipitin reaction.

Provided that one has a single antigen-antibody flocculation reaction with a broad equivalence zone and that non-specific precipitation is not occurring, the calculation of the percentage of given antigen in the preparation under analysis is an obligatory deduction. One of the arguments against this calculation, as advanced by Pope *et al.*, is that the purity of their best preparations of toxin or toxoid, in flocculation units (Lf/mg N) is greater than the value of 2,170 predicted by Pappenheimer and Robinson (16) from their analysis of the flocculation reaction. The outsider to this discussion might think that the flocculation unit (Lf) is akin to a physical constant such as the value for the speed of light. The unit depends, rather, on comparison of given horse antitoxin, designated by international agreement as *a standard* with another horse antitoxin intended for the routine analytical work. The in vitro comparison (optimal flocculation against a given toxin) is subject to significant errors of technique and to the danger of choosing a serum in which the flocculation of unrelated systems masks the reaction of the toxin, as is discussed by Pope. The in vivo comparison has been well analyzed by Jerne (81), whose conclusion is quoted: "Standardization of antitoxin is shown to have been based on false assumptions. The authority bestowed upon the International unit for diphtheria antitoxin has retarded the development of science in this field." This author points out that 2 antitoxins could have the same unitage as determined by flocculation reaction and differ markedly when compared by skin neutralization test (in vivo), and vice versa.

To convince oneself as to how variable is the unitage, it is necessary only to send the same toxin preparation to a number of laboratories and ask them to determine the Lf/mg N of the preparation. In our experience, variations of the order of magnitude involved here have been obtained. In fact, the most valid method available, as pointed out by Pappenheimer, is that of selecting antitoxins which show the "ideal" flocculation reaction and employing them to standardize toxins in terms of specifically precipitable nitrogen. It is highly probable that the difference between the predicted value (Lf/mg N) of purified diphtheria toxin and the higher values found by other workers may be a question only of how the unitage of the various antitoxins employed was determined. The real question here is not how many Lf/mg N are possessed by a purified toxin, but rather what percentage of a given preparation is specifically precipitable as toxin. If the problem is to be decided of how many Lf/mg N the purified toxin possesses, all workers must use the same antitoxin and agree on its unitage. It is obvious that if the predicted value were lower than the value determined for purified toxin and this discrepancy is not due to errors of unitage, one would be forced to conclude that toxin was synthesized during the course of its fractionation or that in the flocculation reaction more toxin is specifically precipitated than is added. Pope's own data support the view that these differences are simply due to the designation of units/ml for the 2 antitoxins used, i.e., Pappenheimer's in 1937 and Pope's in 1951. With 3 different pepsin-treated antitoxins and Pappenheimer's purified toxin, Pope *et al.* determined the N content/toxin-antitoxin unit by analyzing the specific precipitate formed at the point of optimal flocculation. They found 22.3, 23.7 and 21.3 × 10^{-4} mg N, respectively, with these 3 antitoxic preparations and point out that the value is quite different from the value of 16.1 × 10^{-4} mg N reported by Pappenheimer and Robinson. The excellent agreement between the values found with the first 3 antitoxins by Pope *et al.* would be unlikely if appreciable amounts of unrelated specific precipitation or nonspecific precipitation had taken place at the point of optimal flocculation, for the chances of having 3 horses respond identically to all antigenic components is small. Therefore, with these 3 pepsin-treated antitoxins, Pope *et al.* were probably studying a single antigen-antibody reaction. For this reason, the likely explanation of the different results, Pappenheimer's and Pope's, is that of differences in unitage.‡

Furthermore, the sample of Pappenheimer's purified toxin analyzed by Pope, had been sent to him in lyophilized form in 1937. Since the activity of lyophilized toxin is known to fall off unless dissolved quickly after opening, it is difficult to know what changes could have occurred in the preparation. In carrying out the analysis of TN/unit at the point of optimal flocculation, Pope, however, used the value 2,170 Lf/mg N determined in 1937 by Pappenheimer for this preparation.

In a personal communication, Pappenheimer states that he has retitrated recently 1 of the antitoxins extensively studied in 1939 against a new standard flocculating antitoxin and found that the old serum had apparently gained 25 per cent in unitage. In the light of these findings, it would be of great interest to know the value (Lf/mg N) determined by Pope for the purified toxin of Pappenheimer.

Changes in purified toxin on reconstitution following storage in the dried state, therefore, in addition to uncertainties in the unitage of different antitoxins, might in themselves account for the discrepancies between the value (Lf/mg N) assigned to purified toxin by Pappenheimer and Robinson and the results reported by Pope *et al.*

Continuing their argument, Pope *et al.* claim that with certain of their antitoxins, Pappenheimer's purified toxin showed 14 components when tested by the technique of precipitation after diffusion in a gel. This toxin preparation had been analyzed to be 93.5 per cent pure by use of the flocculation reaction (14); i.e., 93.5 per cent of the preparation was specifically precipitable by antitoxin. If, as Pope has done, the precipitation in gels is carried out with an antiserum in which most of the antibody is directed against the atoxic proteins, it is quite possible that one would see the 13 different bands corresponding to the reactions of the same number of antigens in the 6.5 per cent of impurities. The number of boundaries is irrelevant to the question of the percentage of toxin in the preparation. For example, as an extreme case, if one had antibody only against the atoxic proteins, and a toxic preparation of 99 per cent purity was tested against it in a system in gels, one might see 13 antigen-antibody reactions, none of which is due to the major component, the toxin, and conclude, therefore, that the preparation was grossly impure. Bowen (82) recently reinvestigated the reaction between toxin and horse antitoxin using the technique of diffusion in gels. He confirmed Pope's observations that many sera do possess detectable antibody against atoxic proteins. However, he carried the observation further by attempting a semiquantitative analysis of the system. Bowen demonstrated clearly that by using very high concentrations of toxin (1,000–3,000 Lf/ml), as Pope has done, one can demonstrate a large number of reactions due to atoxic protein impurities in the purified preparations, but that these impurities may amount to a very small percentage of the total antigenic material present.

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‡ One other fact is important to note in addition to the question of units. Another difference in the results of Pope and Pappenheimer could be due to the method and extent of peptic treatment of the antitoxins. From the point of view of this discussion it would have been more pertinent had Pope compared untreated antitoxins. Pappenheimer and Robinson showed that with 4 different untreated antitoxins and 3 different toxin preparations, they obtained identical values of TN/unit antitoxin. The 1 different result obtained was with a commercial, pepsin-treated, antitoxic globulin, and it seems incorrect to compare quantitatively this commercial preparation with 3 others obtained 14 years later, especially when the techniques of preparation and extent of digestion are unstated.

The findings reported by Pope *et al.* on precipitation in gels have not been uniformly encountered. For example, Oudin, in the chapter which follows, discusses the reaction in gels between diphtheria toxoid and horse antitoxin prepared at the Pasteur Institute and reports the finding of a single boundary. With certain antitoxins Ouchterlony (83) found 3 boundaries, 1 distinctly due to the toxin itself and the other 2, very faint, due to unrelated systems. The author, in an unpublished investigation of atoxic diphtheria proteins, tested about 30 high titer horse antitoxins prepared commercially in the United States but did not find one with sufficient antibody against the atoxic proteins to be useful in following the isolation of these antigens. Pope *et al.* apparently have worked with sera which contain larger amounts of antibody against the atoxic proteins. These complex systems, however interesting, cannot be used to analyze (1) the properties of the flocculation reaction or (2) the purity of various toxin preparations. Simpler systems must be looked for and employed.

Pope *et al.* remark that their observations show that diphtheria toxin has not been obtained as a pure substance. If multiple, rigid criteria are applied, the same can be said for most highly purified proteins. In any case the present author is of the opinion that the preparations studied by Pappenheimer were sufficiently pure to enable a valid determination of molecular weight and chemical composition to be made, and that these determinations are not invalidated by more recent observations. In addition, Pope *et al.* were apparently unaware of the work of Agner (84), who confirmed Pappenheimer's observations on the purification of toxin. Agner isolated a toxin preparation at least 99 per cent specifically precipitable, which moved as a single boundary on electrophoresis and which had toxic activity of 55 MLD/Lf and 2,200 Lf/mg N. Pope *et al.* have used immunologic criteria only and have not introduced measurement of toxicity in their analyses. Finally, it is appropriate to point out that toxoid preparations have been obtained which are pure by all existing physical criteria (solubility, electrophoresis, etc.) and which contain 2,100–2,200 Lf/mg N comparable to the equivalent toxin preparations (85). Since toxin and toxoid are immunologically indistinguishable, there is no reason to expect that isolated samples of toxin will possess values of Lf/mg N markedly different from the corresponding toxoid.

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B. Specific Precipitation in Gels and Its Application to Immunochemical Analysis*

J. OUDIN, *Institut Pasteur, Paris*

Bechhold in 1905 (1) studied the reaction between antigens and antibodies in gels, reactants whose property of forming a precipitate had been known since the work of Krauss in 1897. While working with goat serum and its antibodies, the former observed the formation of ring structures in his tubes which resembled Liesegang rings, a phenomenon first observed in 1896 with mineral reactants. Reiner and Kopp in 1927 (36) also described what they believed was Liesegang ring formation during specific precipitation.

Since 1932, specific precipitation in gelified media has been used for bacteriologic studies; Sia and Chung (37) used a technique for the identification of pneumococcus types in which an opaque ring developed around colonies grown on layers of agar containing the homologous antiserum. At almost the same time Petrie (34) made more detailed observations when he attempted the transformation of "smooth" into "rough" bacterial types by culturing them on media containing antiserum. The specific halos which formed around meningococcus, pneumococcus and *Shigella dysenteriae Shiga* colonies often had a uniform appearance at the start, only to assume at a later stage the form of Liesegang rings; a similar type of precipitation was observed when solutions of the corresponding polysaccharides diffused into the same gel. Maegraith (14), Kirkbride and Cohen (13) and Petrie and Steabben (35) applied similar techniques to the study of meningococcus and to the identification of gas gangrene anaerobes.† These investigations accomplished their immediate bacteriologic purpose, and the occasional observation of concentric rings was only an accessory result vaguely classified as the Liesegang phenomenon or mentioned without attempt at interpretation. The interpretation of concentric ring formation as the manifestations of several antigens was not considered.‡

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The qualitative analysis of a naturally occurring mixture of antigens presents a problem whose resolution has always necessitated a combination of physicochemical methods, such as salting out, with the methods of immunochemistry and antibody absorption. Fractionation has proved necessary, since the classic immunochemical methods (those of Ramon, of Dean and Weber and of Heidelberger and Kendall) are essentially methods for titration of a given antigen or antibody and may be most successfully applied under conditions where only a single antigen-antibody reaction obtains.

However, molecules (particularly protein molecules) endowed with the same immunologic specificity are generally better characterized by this specificity than are molecules having the same solubility in a given solvent or any other common physical property. Moreover, the immunologic specificity of proteins is related to their precise biologic origin. It should therefore be possible to effect the analysis of naturally occurring mixtures of antigens without recourse to physical methods.

In this chapter we shall use the term "immunochemical analysis" to denote the determination without resort to previous physicochemical fractionation of the number of antigens present in a mixture and their identification. In seeking a solution to the problem thus defined, it seemed to us that if the effect of gravity could be eliminated in carrying out the precipitation reaction at an interface, each antigen-antibody system present might give rise to a specific zone of precipitation. This hypothesis was verified by incorporation of one of the specific reactants in a gel. The 1st results using this technique were published in 1946 (27, 28) and showed that under appropriate conditions: (1) a single antigen gave rise to only 1 zone of precipitation, and (2) when 2 distinct antigens were present in the same solution, each behaved independently as though the other were not present.

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† The earlier work of Nicolle *et al.* (18) and the more recent studies of Hanks (10), sometimes cited in this connection, are in reality unrelated to the present subject despite the fact that the authors used gelatin.

‡ A multiplicity of antigens may not be the only explanation in every case and in particular appears insufficient to explain the observations of Brown (see note, p. 355) on the rhythmic precipitation of the pneumococcus polysaccharides with antipneumococcus serum (4).

Thus specific precipitation during the course of diffusion through gels constitutes the basis for a general method of immunochemical analysis. The techniques which have been described are conveniently classified as techniques of simple diffusion and of double diffusion.

1. In our 1st paper (27) a technique for simple diffusion in 1 dimension (in tubes)§ was briefly described, discussed and applied to the analysis of antigens in horse serum, and double diffusion (in tubes) was suggested as a possibility.

2. Techniques of double diffusion in 2 dimensions (using agar plates) were developed by Ouchterlony (19) and by Elek (7) to study the toxin production by bacteria. Ouchterlony studied, in various ways, the precipitation reaction in gels between diffusing substances, derived either from whole cultures or from toxin-containing filtrates, and immune sera. Elek, on the other hand, cultured different strains of *Corynebacterium diphtheriae* and of staphylococcus in agar plates through which the constituents of the antiserum diffused. Both workers observed multiple zones of precipitation which they considered might have been caused by a multiplicity of antigens. Subsequently they applied their respective techniques to the analysis of mixtures of antigens. Since 1946, the Liesegang phenomenon does not seem to have been mentioned in connection with antigen-antibody reactions in gels.

THEORETICAL BACKGROUND

The following discussion is concerned with the 2 phenomena associated in *specific precipitation* during the course of *diffusion* through a gel.

DIFFUSION (6, 11).—If a given solution and the corresponding pure solvent are layered 1 over the other without mixing in a vertical tube, molecular agitation will result in penetration of molecules of solute into the solvent layer.

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§ Munoz and Becker (17) have confirmed the validity of this technique, which has been applied by them and a certain number of others.

When the concentration of solute is sufficiently low so that intermolecular action is negligible, the process is described in a general way by Fick's law which relates the concentration c of the diffusing substance at any level x and time t

$$\left(\frac{dc}{dt}\right)_x = D\left(\frac{d^2c}{dx^2}\right)_t \qquad (1)$$

where D is the diffusion coefficient of the diffusing substance.

Integration of this equation leads to the following:

1. When diffusion takes place from a solution of constant concentration, Stefan has established a relationship between the initial concentration c_0 and the concentration c at time t of the diffusing substance of diffusion coefficient D at a point in the column of solvent at distance x from the interface of a solution-solvent

$$c = c_0\left(1 - \frac{2}{\sqrt{\pi}}\int_0^y e^{-y^2}\,dy\right) \qquad (2)$$

where $y = \dfrac{x}{2\sqrt{Dt}}$.

2. In the case of a column of infinite length on both sides of the initial interface between solution and solvent

$$c = \frac{c_0}{2}\left(1 - \frac{2}{\sqrt{\pi}}\int_0^y e^{-y^2}\,dy\right) \qquad (3)$$

Equation (3) differs from equation (2) only by the coefficient $1/2$ which affects the right-hand side of the equation. The equations are represented by curves shown in Figures 1, 18, 21 and 22.

In the case of equation (3), the concentration at the level of the initial interface is constant and equal to $\frac{c_0}{2}$ for all values of $t > 0$. It follows from the preceding equations that for any concentration $c_i < c_0$ in equation (2) and $c_i < \frac{c_0}{2}$ in equation (3), at a point x where the concentration will always be equal to c_i, one obtains for all values of t

$$\frac{x_i}{\sqrt{t}} = c^t \qquad (4)$$

It should be added at this point that coefficients of diffusion increase with temperature.

SPECIFIC PRECIPITATION[‖].—The different precipitating systems can be classed as follows: (1) *Simple precipitating systems* in which a single antigen reacts with an antibody. In this case we are not concerned with the presence or absence of other antigens or of other antibodies which do not enter into the reaction or with whether or not the reaction is homologous. (2) *Complex precipitating systems* in which several antigens cross-react with a given antibody, e.g., the reactions between the ovalbumins of chicken and duck with anti-chicken ovalbumin serum. (3) *Multiple precipitating systems* consisting of antigens and antibodies reacting simultaneously which may be components of several simple or complex precipitating systems.

There exists for each of the 2 reactants, antigen and antibody, a minimum concentration below which precipitation is not visible. As discussed by Cohn in the preceding chapter, the composition of any precipitate is determined by the ratio of the concentrations of antigen to antibody in the mixture, and there exists a *ratio of equivalence* at which both reactants are completely precipitated. The specific complex is soluble in excess of antigen; in fact, one can take a washed specific precipitate formed at the equivalence point and dissolve it more or less completely in excess antigen. In the case of the flocculation reaction ("horse" type), the specific complex is also soluble in excess antibody, in contrast to the precipitin reaction ("rabbit" type). Because we will have occasion to refer to this later, it should be recalled that in both cases maximum total precipitation occurs at the equivalence ratio or in a slight excess of antigen when antibody remains constant (pp. 314 ff.).

The reaction of precipitation occurs in 2 steps: (1) combination of antigen and antibody, and (2) aggregation leading to a precipitation.

The time for the 1st stage is very short and in 1 instance has been shown to be less than 3 sec (15). On the other hand, the 2d stage often takes an appreciable length of time which varies, depending on the ratio of concentration of the reactants. The optimal precipitation titrations of Ramon and of Dean and Webb depend on the time differential in precipitation. For the flocculation reaction, the most rapid visible precipitation generally occurs at a point in the middle of the equivalence zone, and for the precipitin reaction at or near the equivalence ratio.

SIMPLE DIFFUSION

Under this imperfect but convenient title we will include those cases in which 1 of the reactants, i.e., antigen or antibody, diffuses

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[‖] See Cohn, "Reaction in Liquid Media," this volume.

into a gel containing, at the beginning of the experiment, the same concentration of the other reactant, i.e., antibody or antigen, at every point in the gel. The latter reactant will be called "internal" reactant and the former, the "external" or "diffusing" reactant, according to the terminology adopted by others who have used analogous techniques with inorganic substances (38).

Agar has been almost the only substance used for preparation of gels. Gelatin has limited usefulness because the temperature at which it liquefies is too low.

SIMPLE DIFFUSION IN 1 DIMENSION (27–33, 17, 2, 3)

TECHNIQUE.—*Preparation of agar:* A given weight of the purest agar obtainable is cut very fine and washed several times with distilled water, after which the pH should be close to neutrality. The agar is suspended in distilled water in 4–5 per cent concentration by weight and placed in a boiling water bath until dissolved, then autoclaved for 15 min at 115 C. The solution is clarified while hot either by centrifuging or, more simply, by filtration through double sheets of fluted coarse filter paper.* The agar should be perfectly limpid while in the liquid state.

The concentration of agar always decreases during this procedure. Therefore the dry weight is determined and distilled water added so as to obtain, for example, a concentration of 2.4 per cent—a percentage high enough to allow for dilution with the internal reactant. Solid NaCl is added to final concentration of 0.85 per cent.

For future preservation and use, known volumes of the agar are distributed in glass tubes which are sealed by flame and sterilized twice at 1-day intervals by heating at 100 C for 30 min in an open autoclave.

Preparation of tubes: The width of tube chosen depends to a certain extent on the amount of material at one's disposal. There is no reason to choose a tube of greater than 4 mm I.D. One can, with a bit of experience, use tubes of 1.5 mm I.D. In our quantitative studies (31, 32) we have used tubes of 3 mm I.D. If one wishes to make microphotometric recordings, it is convenient to use cells with parallel walls, e.g., having 1.5 × 5 mm cross-section (cells with these dimensions were used in the reactions illustrated in Figs. 2–4, 6, 7, 9, 15 and 16).

Normally, the agar will not stick to glass, so to prevent the external reactant from flowing into the space between gel and wall, the tubes are treated in the following manner. They are heated to 60–70 C and filled with 0.6–1.0 per cent agar solution at the same temperature. They are immediately emptied and plunged into an ice bath; their inner surface thus becomes coated with agar, which is dried by placing the tubes in a vacuum desiccator over sulfuric acid or phosphoric anhydride for 2 or 3 days. This coating can be detected by its opalescence, which disappears as soon as a solution of agar is added. The coated tubes may be used for several months.

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* In our laboratory, Chardin paper is used.

In nearly every case we have used a final concentration of 0.3 per cent agar in mixture with internal reactant. This low concentration of agar results in a limpid wet gel which has the consistency required when narrow tubes are used (a higher concentration could naturally be chosen). For most work the agar solution at 0.6 per cent is diluted in equal proportions with internal reactant. However when minimal dilution is desired, the original 2.4 per cent agar solution may be mixed with as much as 7 vol of the internal reactant. It is important to add a convenient antiseptic, e.g., Merthiolate in concentration of 2×10^{-4} by weight.

The original agar solution is melted in boiling water, then placed in a bath at 46–48 C and mixed at this temperature with the internal reactant, previously clarified with care, e.g., by centrifugation, and brought to the same temperature.

If the internal reactant is an antiserum to be used at different concentrations in several tubes, it is often preferable to have the total concentration of serum the same in all tubes; to do this, the antiserum is diluted in the serum of an unimmunized animal of the same species. For quantitative observations, this precaution is taken in order to have the concentration of nonspecific substances roughly the same in the gel (see p. 351).

The reactants are distributed in the coated tubes with a Pasteur pipet whose delivery is controlled by a rubber tube to the mouth. For the height of the column of gel, 35–45 mm is convenient. So that the meniscus of the interface will be of relatively constant shape in all tubes, it is best to fill them to a level several mm higher than that finally desired and immediately withdraw the excess. After distribution of the agar mixture (at 46–48 C) the tubes are placed at the temperature of the experiment. Once the gel has solidified the external reactant is added in as high a concentration as possible and at the same temperature to approximately constant height. A 10–20 mm height can be used if necessary, but 30–35 mm is preferable. It may be convenient, at times, to incorporate both layers in gelified media (p. 351).

The tubes may be sealed with rubber caps or with a mixture of wax and tar or with modeling clay, etc., to prevent evaporation.

It is best to keep the tubes at approximately constant temperature, and, above all, sharp changes of temperature should be avoided (see p. 355). A simple way to avoid artefacts due to changes of temperature is to enclose the tubes in a small container (a box or glass jar) lined with a heavy layer of cotton and not to remove them until the moment chosen for their examination, e.g., at the end of 4 or 7 days.

In this laboratory, observations are habitually made within 1 week after commencement of the experiment. When a rapid answer is necessary, the essential data can often be obtained in 1 or 2 days. The tubes may be read against a totally black background with side-lighting or against a uniformly lighted background.

A simple and inexpensive system allows one to photograph as many as 13 tubes of 2 mm I.D. at 1 time with a 2-fold enlargement on a photographic plate 9 × 12 cm (Fig. 17). The lighting is furnished by 2 broad horizontal light sources placed between the tubes and the black background at equal and constant distances above and below the axis of the objective. The sharper the angle formed between the line from light source to the object and the axis of the camera objective, i.e., the farther the light sources are from the tubes, the more readily will zones of weak precipitation or of small particles be photographed (Fig. 9 could have been taken only in this way). The wider the angle, i.e., the closer the light sources are to the tubes, the more readily may boundaries situated close to one another be distinguished.

For quantitative studies, it is necessary to measure penetration h (see p. 348). In this laboratory these measurements were made at first by means of an apparatus built specially for this purpose (32). More simply, and with apparently sufficient precision, Munoz and Becker (17) use calipers. The measurements are now made by the author from photographs on glass obtained directly with a 2× enlargement.

THEORETICAL DISCUSSION.—*Density of precipitate* will be defined as the quantity of precipitate/unit vol, where unit vol denotes the smallest volume whose content or precipitate may be considered homogeneous. For lack of a simple and rapid method of measuring the density of precipitate, we must be satisfied with a comparison between different points in the same gel. It is assumed as probable, within certain limits, that the point of maximum light scattering by the precipitate coincides with the point of maximum density of precipitate.†

The density of precipitate at any given point in the gel is determined by a series of complex relationships which are linked, on one hand, to phenomena of diffusion and, on the other, to immunochemical phenomena, not to mention quantitative changes due to the gelling substance itself and to particular properties of gels.

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† Above a certain value for the density of precipitate this ceases to be true; in this rare case, the precipitate absorbs light more than it scatters it, and the region of precipitation does not appear illuminated.

Simplifying Hypotheses.—The following simplifications allow us to describe approximately the phenomena on which the method depends.

A gelling substance is employed to avoid the effect of gravity and mechanical agitation which are unavoidable in liquid solutions. It is assumed that its presence will not bring about any other qualitative changes. In most cases the density of precipitate at each point in the gel is considered to be determined by the concentration of the 2 reactants at the same points, in accordance with the laws which determine (at the end of an appreciable time) the formation of a specific precipitate in liquid medium using the same reactants at the same concentrations.‡

The foregoing assumptions neglect 2 facts:

1. The formation of a specific precipitate is not instantaneous; therefore, if the concentration of antibody at any given point varies but slightly and the concentration of antigen changes rapidly from 0 to a value high enough to inhibit precipitation (during a period too short for the precipitate to form), precipitation will never appear at this point even though the reactants have doubtless combined. If one can consider the antigen-antibody combination as instantaneous, the absence of precipitation under certain conditions (always close to the source of diffusion of the antigen in precipitin-type systems) does not appear to change the outcome of the experiment. More generally, by neglecting the time of formation of the precipitate, one overevaluates the time required for a given concentration of the diffusing reactant to reach a given point in the gel, but ordinarily this error is negligible. To avoid this minor difficulty in our theoretical discussion the *equivalence point* will be referred to as the point or level in the gel where the concentrations of antigen and antibody are in their ratio of equivalence at the moment under consideration. It is clear that the equivalence point should lie close to the point of maximal density of precipitate when the internal reactant is antibody, if precipitation were instantaneous.

2. When the point of equivalence migrates in the gel, antigen-antibody complexes will be formed (depending on the point considered in the gel) either by the direct combination of the 2 re-

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‡ This hypothesis is necessary to describe reactions in gels in terms of the known facts concerning reactions taking place in liquid solution. The hypothesis has been confirmed satisfactorily by experiment not only for nearly all cases of simple diffusion studied but also for double diffusion (cf. Figs. 21 and 22 with 23 and 24). The Liesegang phenomenon will not be considered in this discussion.

actants or by a new reaction of 1 or the other reactant with the complexes already formed.

In conclusion, the simplifications lead us (1) to consider as instantaneous the initial formation of the specific precipitate and its subsequent reactions, and (2) to suppose that the final density of precipitate is always the same for given final concentrations of the 2 reactants.

The case of simple diffusion will be treated as if the internal reactant itself does not diffuse when precipitation occurs in the gel. This is evidently not exact since molecular agitation, the cause of diffusion, involves the molecules of the internal reactant as well as those of the external reactant,§ but the resulting variations in concentration of the internal reactant in the agar layer are slight compared with those of the external reactant, as has been verified by experiment (Fig. 4).

It will be recalled that, under the conditions for applying equations (2) and (3), the concentration of diffusing substance remains constant in the immediate neighborhood of the interface once diffusion has commenced. It will be assumed that this constancy is realized. Even though the experimental conditions deviate from the ideal, certain predictions of the above theoretical equation may be verified satisfactorily by experiment (see p. 347 and Fig. 5). It is quite possible that this confirmation is due partly to a compensation between the errors resulting from diffusion of the internal reactant and from changes in concentration of external reactant at the interface.

Theoretical Analysis of Simple Diffusion.—The conditions for simple diffusion are apparently quite different from those for free diffusion, above all when the ratio of initial concentration of the 2 reactants approaches their equivalence ratio. In the particular instance in which the ratio of initial concentration is that of equivalence, precipitation occurs in the immediate vicinity of the interface and neither of the 2 reactants penetrates to any appreciable extent into the layer containing the other reactant. On the other hand, when the concentration of internal reactant is very low with respect to the initial concentration of the external reactant, the latter's concentration at different levels in the gel will approach that expected in free diffusion, at least up to the point in the gel where the equivalence ratio of the 2 reactants becomes realized.‖

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§ Certain artefacts mentioned later are caused in part by diffusion of the internal reactant (see p. 355).

‖ Becker *et al.* (2, 3) have recently called attention to this limiting case and to the possibility of applying the laws of free diffusion to it (see p. 351).

Between these 2 extremes (Fig. 1) the concentration of external reactant at different levels in the gel cannot be determined with certainty from the laws of free diffusion. However, it follows from these laws that for a concentration of external reactant which remains constant at the level of the interface, and for a concentration of internal reactant which remains constant throughout the gel, the distance from the interface to the point of equivalence is proportional to the square root of time (equation (4)).

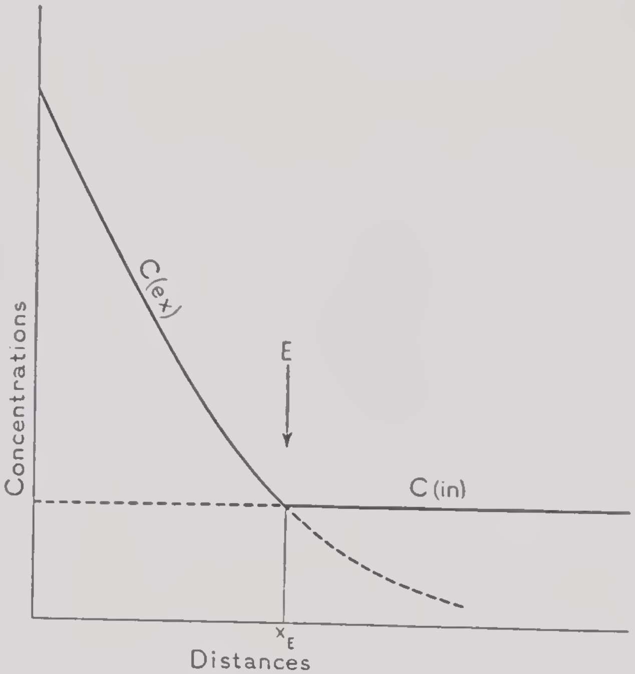


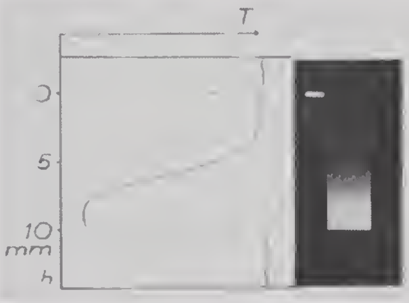
FIG. 1.—Diagram representing simple diffusion at a given time t, in conformity with simplifying hypotheses discussed in the text. Concentrations of external reactant $C(ex)$ and of internal reactant $C(in)$ are expressed in equivalent units, e.g., flocculating units of toxin and antitoxin; therefore equivalence point (E) corresponds to the point of intersection of the 2 curves. $C(ex)$ curve has been drawn following theoretical equations of free diffusion, justifiable only when $C(in)$ is negligible with respect to initial values of $C(ex)$. For this reason, the dash-line portion of this curve does not have precise significance. Assuming $C(in)$ to be constant for all values of x and t, x_E is proportional to $\sqrt{t}$. In the broken portion of the curve $C(in)$, the internal reactant has combined with the external reactant.

Moreover, if the concentration of the internal reactant remains constant at all levels of the gel, the density of precipitate at the equivalence level should also remain constant provided the time of formation of precipitate may be neglected.

In practice, one nearly always chooses antibody as internal reactant because of the properties of precipitin-type systems (see p. 347 and Figs. 2 and 3).

Theoretical Basis for Determination of Number of Reacting Antigens.—It has been assumed that the concentration of the internal reactant remains constant in the column of gel. If a single antigen is the diffusing reactant, the curve describing the density of precipitate at a given moment as a function of distance from the interface will pass through 1 and only 1 maximum.

This is to be expected, considering the known properties of the curve describing the amount of precipitate obtained as a function of antigen added to a constant amount of antibody, according to the quantitative method of Heidelberger *et al.* Aside from the simplified assumptions introduced above for consideration of specific precipitation in tubes of gel, the only difference between reactions occurring in gels and those occurring in liquid solutions resides in the fact that, in the former case, one does not know exactly what relationship describes the variation in the concentration of antigen as one passes from the point farthest from the interface to the interface itself. However, it is known that this concentration increases in a continuous manner, and this fact alone leads to the conclusion that in the case of a single antigen the density of precipitate will never pass through more than 1 maximum. Therefore the number of antigens reacting in a system cannot be less than the number of observed maxima of density of precipitate.



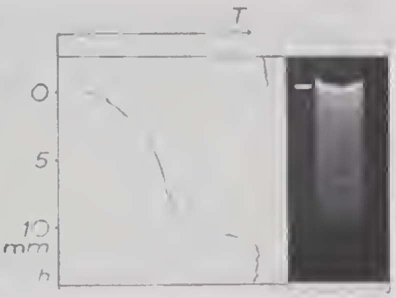


Fig. 2 (*left*).—Reaction of chicken ovalbumin (reactant diffusing from upper liquid layer) with homologous rabbit antiserum (lower gellified layer). Microphotometric tracing was made from a photographic negative. Ordinates (distances from each given level to the interface) are in the same scale for the tracing as for the photograph of the cell in which the reaction took place. Abscissae are assumed to be proportional to $T = I/I_0$ (I = light transmitted to photoelectric cell of microphotometer; I_0 = light emitted). Interface is marked on the photograph by a white dash and recorded on top of the tracing by a peak. The leading edge of the zone of precipitation is very sharp. The density of the precipitate passes through a maximum in the neighborhood of this limit (29, 32).

Fig. 3 (*right*).—Antibody is the diffusing reactant (upper layer: anti-ovalbumin rabbit serum); antigen is in the gel in low concentration. Density of precipitate increases from bottom to top until the interface is reached, since the excess antibody does not dissolve the precipitate (29, 32).

Experimental Examples.—simple precipitating systems.—The simple system that has been most thoroughly studied is that of chicken ovalbumin and its homologous rabbit antiserum.

1. When ovalbumin diffuses into a gel containing homologous antibody (Fig. 2) the zone of precipitation* has a sharp leading edge, while its upper part containing antigen-antibody complex partially or completely dissolved in excess antigen is diffuse. The density of precipitate passes through a maximum close to the leading edge.

2. When rabbit antibody diffuses into a gel containing the antigen the density of precipitate increases without passing through a maximum anywhere between the lowest point of the zone and the interface, because of the absence of inhibition by excess of antibody (Fig. 3). For this reason the choice of antigen as diffusing reactant is preferable for the purposes of immunochemical analysis.

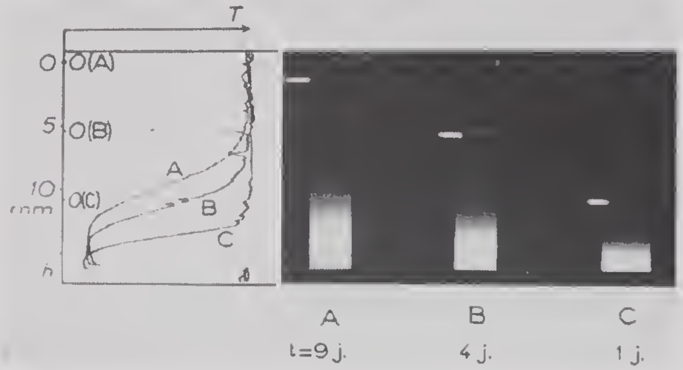


FIG. 4.—Development in time t of the zone of precipitation of chicken ovalbumin (external reactant) with homologous rabbit antiserum (internal reactant), with corresponding microphotometric tracings (see legend, Fig. 2). Same initial concentrations of the 2 reactants in all 3 cells. Each white dash indicates level of the interface (29, 30, 32). j = days.

Influence of the Principal Variables.—Time: The maximum density of precipitate changes only slightly during the time it takes for the leading edge to migrate from the interface into the gel (Fig. 4). The leading edge formed by precipitation of the diffusing reactant, ovalbumin, is very sharp. The leading edge is at first convex, like the interface, but after several hours tends to flatten out and form a horizontal plane. The displacement or penetration, h, of the leading edge can then be measured with a precision of 0.1 mm, and is a linear function of the square root of time t,† as predicted by the theory; but h at 0 time, as determined by extrapolation, is generally situated slightly above the interface, about 0.3 mm on the average in the experiments cited here (Fig. 5).

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* We have chosen the term "zone of precipitation" instead of "zone of precipitate" to emphasize the fact that the precipitate itself does not migrate although the precipitation takes place at points in the gel farther and farther from the interface.

† In 1888 Chabry (see reference in Duclaux (6)) found that $h/\sqrt{t}$ remained nearly constant (as predicted by Fick's law) in experiments on diffusion of an acid in columns of liquid, of gelatin and of hyaline cartilage containing the indicator orcein.

Concentration of the 2 reactants: Experiments designed to study the influence of concentration on the maximum density of precipitate are illustrated in Figures 6 and 7. These figures demonstrate that the initial concentration of antigen (diffusing reactant) does not significantly influence the maximum density of precipitate. The maximum density, on the other hand, varies considerably with the concentration of antibody (initial reactant). We would suspect a priori an approximate proportionality between the maximum density and the concentration of internal reactant, provided the smallest concentration of antibody which gives a visible zone is negligible compared with the amount of antibody actually reacting.

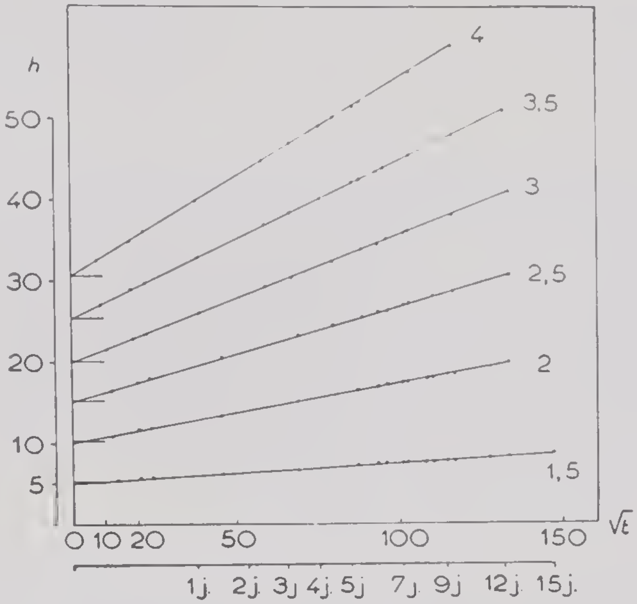


Fig. 5.—Measured distances (mm) of boundary of precipitation of ovalbumin from an arbitrary starting point plotted as function of the square root of time t (min) for 6 tubes. Each horizontal dash along the ordinate h indicates the level of the interface in each tube. Initial concentration of the internal reactant (antiserum) was equal in all tubes; figures on the right are logarithms of initial concentrations of antigen (32).

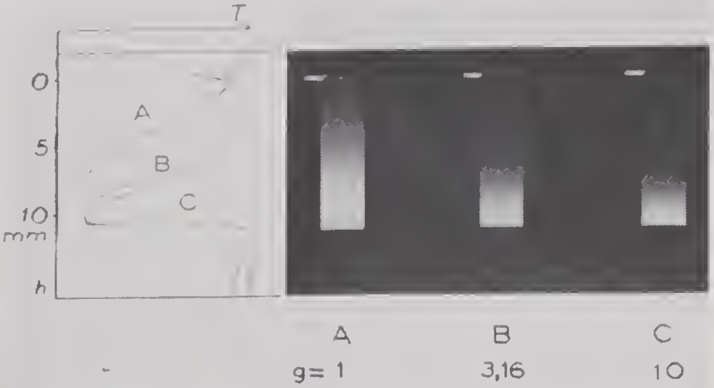
The influence of antigen and antibody concentrations on the penetration, h, is expressed in approximate fashion by the following relationships which have been established empirically for the case of a single antigen (31, 32).

1. For each given value of the initial concentration a of the antibody

$$\frac{h}{\sqrt{t}} = \gamma \log \frac{g}{g_0} \tag{5}$$

where h is the distance from the interface to the leading edge, t

the time of diffusion, g the initial concentration of the antigen, g_0 the extrapolated value of g for which $h/\sqrt{t} = 0$, and γ is a coefficient > 0.



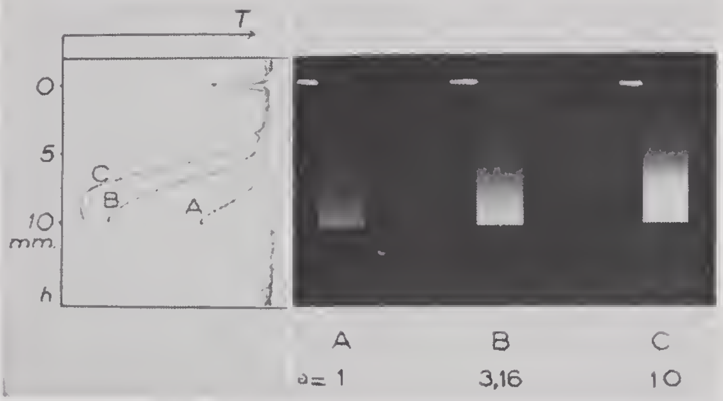


FIG. 6 (*above*).—Photograph with microphotometric tracing (see legend, Fig. 2) showing influence of initial concentration of external reactant (ovalbumin) on zone of precipitation. Same reactants as in Figure 4; initial concentration of immune serum is equal in all 3 cells. Initial concentration of ovalbumin was proportional to values of g. Time was calculated from equation (5) to give approximately the same penetration in all 3 cells at the moment of photographing. Each white dash indicates level of the interface (29, 30, 32).

FIG. 7 (*below*).—Photograph with microphotometric tracing (see legend, Fig. 2) showing influence of initial concentration of antibody (internal reactant) on zone of precipitation. Same reactants as in Figures 4 and 6. Initial concentration of antigen was equal in all 3 cells; initial concentration of antibody was proportional to values of a. Time was calculated from equation (6) for penetration to be approximately the same in all 3 cells at the moment of photographing. Each white dash indicates level of the interface (29, 30, 32).

2. For each given value of g

$$\frac{h}{\sqrt{t}} = \alpha \log \frac{a}{a_0} \tag{6}$$

where a is the initial concentration of antibody, a_0 the extrapolated value of a for which $h/\sqrt{t} = 0$, and α is a coefficient < 0. The values of γ and α depend on the nature of the antigen. $h/\sqrt{t}$ varies in the same direction as g and inversely as a.

By varying the concentration of only 1 reactant at a time, one can represent the variations of $h/\sqrt{t}$ as a function of log g and log a by 2 series of straight lines which, for the experiment cited here, are reproduced in Figure 8. The extrapolation of these lines to 0 for $h/\sqrt{t}$ gives values for g and a whose ratio, except in extreme cases, is approximately that of the equivalence ratio. As will be seen later, equations (5) and (6) cease to hold at high values of $h/\sqrt{t}$.

Coefficients of diffusion: Mitchison and Spicer (16) attempted to apply the theoretical equations to diffusion of antibiotics in tubes of gel. Their calculations, made by means of expansion into series, showed that: (1) in equation (3), for low values, $x/\sqrt{t}$ tends to become a linear function of log c and the expression approaches the empiric equation (5), and (2) when $x/\sqrt{t}$ is large, x^2/t tends to become a linear function of log c.

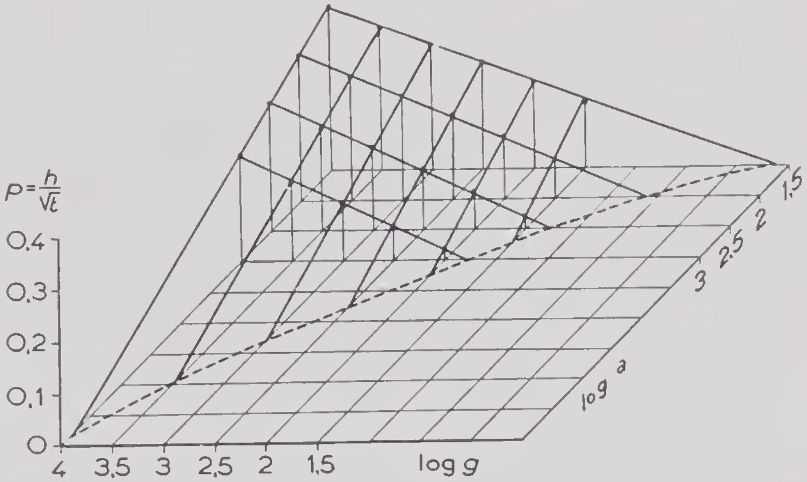


FIG. 8.—Precipitation of ovalbumin in a gel containing immune serum. Figure shows variations in $h/\sqrt{t}$ (h in mm; t in min) as a function of log g and log a, where g and a are initial concentrations of ovalbumin and anti-ovalbumin (31, 32).

The 2d observation, which was verified experimentally in the case of diffusion of antibiotics, has been confirmed by Becker *et al.* (2, 3) for the diffusion of antigens by using the present technique. When g is very high and a very low, they have shown that the slope of the line which represents log g as a function of h^2/t is inversely proportional to the coefficient of diffusion of the antigen; this slope is equal to $\frac{1}{4D}$ log e. They have thus developed a method of determining D in which it is not necessary to use a simple system nor to know the concentration of antigen or antibody. The values for D obtained by this method for several albumins agreed satisfactorily with the accepted values as determined by free diffusion (although slightly lower).

The same authors indicated a 2d method of determining D when g is large and a is small; in other words, the condition which most closely resembles free diffusion. Starting with the hypothesis that at the level h of the leading edge of the zone of precipitation the ratio of concentrations of the 2 reactants has the same value R as in a mixture at equivalence, and granting that the concentration of antibody remains equal to a for all values of h, they substituted in equation (2) the known value of g for c_0, the value of h measured at time t for x and the known value of Ra for c. R is obtained by the classic immunochemical method which requires an antigen of sufficiently high purity, contrary to the preceding means of determination. The values of D found by this means were close to the preceding.

Other Variables: Finally, the influence of other variables should be mentioned briefly. The value of $h/\sqrt{t}$ increases at higher temperatures. If the antigen is not present in pure solution, the value of $h/\sqrt{t}$ may be increased due to the presence of nonspecific substances provided their concentration is sufficiently high.‡ On the other hand, as the concentration of diffusible substances other than the antibody in the gel is increased above a certain value, a diminution of $h/\sqrt{t}$ results. An increase in concentration of agar will also decrease the value of $h/\sqrt{t}$.

From the foregoing discussion the limiting conditions for the appearance of a zone in the gel may be stated as follows: (1) The concentration of antibody must be sufficiently great for the zone of precipitation to be perceptible. (2) The ratio of the initial concentration of antigen to the concentration of antibody must be distinctly greater than their equivalence ratio (see p. 350).

A fairly large number of simple precipitin-type systems has been studied. They have given a single zone of precipitation with a single maximum of density of precipitate.

Most protein antigens show a zone of precipitation with a sharp leading edge. In 2 cases investigated, polysaccharide haptenes gave a zone with a diffuse leading edge (Fig. 9).

Figures 10 and 11 show the type of reaction obtained with flocculating antibody (horse diphtheria antitoxin). When the external reactant in the flocculating system is the antigen (Fig. 10), the result is similar to that obtained between precipitin-type antibody and a protein antigen. On the other hand, when the external reactant is flocculating antibody (Fig. 11), the result is very different from that of precipitin antibody (Fig. 3), due to solubility of the specific complex in excess flocculating antibody. It may be convenient, particularly in such cases as the present one, to incorporate both reactants in layers of gel, so that the zone of precipitation may be observed in either layer, depending on the ratio of the initial concentrations of antigen and antibody.

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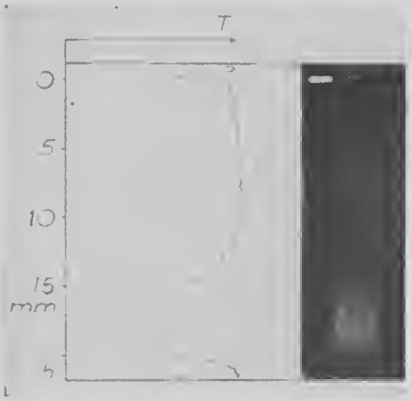
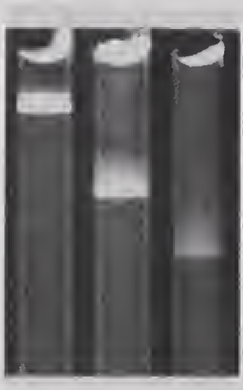
‡ This implies the following practical consequence. If 2 solutions of the same simple antigen react in 2 different tubes of the same gel to give identical values of $h/\sqrt{t}$, it follows that the concentration of antigen is the same in both solutions. If 1 of the solutions contains the antigen in crude mixture with other substances, then for equal penetration h the concentration of the pure antigen in solution will be higher than or equal to its concentration in the crude mixture. The relation between $h/\sqrt{t}$ and the concentration of nonspecific substances does not appear to follow any simple general law. This problem is under investigation.





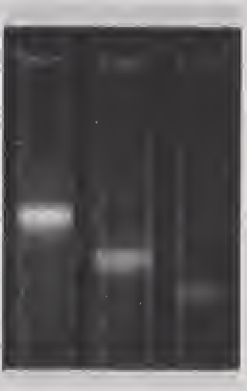


FIG. 9 (*left*).—Photograph and microphotometric tracing (see legend, Fig. 2) of zone of precipitation of the O polysaccharide of *Eberthella typhosa* (external reactant) with an antisomatic serum. This figure contrasts with those of the reaction of ovalbumin by absence of a sharp boundary (29, 32).

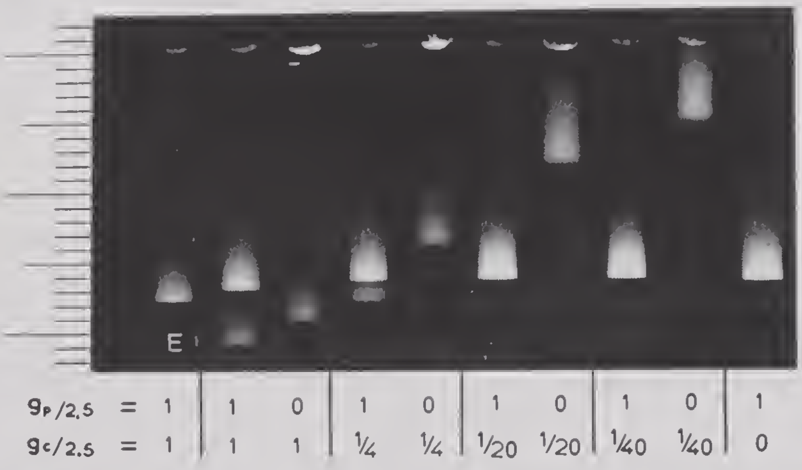
FIG. 10 (*center*).—Zones of precipitation of purified diphtheria toxoid (external reactant) with horse antitoxin (internal reactant) at varying initial concentrations of serum in proportions of 1, $^1/_3$, $^1/_{10}$. Appearance of the zones resembles those of Figure 2 with rabbit antiserum as internal reactant (33). The toxoid used in experiments for Figures 10, 11 and 19, *D*, was a highly purified product prepared by Dr. L. Pillemer; its activity, determined by Dr. Melvin Cohn, was 2,150 Lf/mg N. The antitoxin, supplied by Dr. LeMétayer, contained 1,600 units/cc.

FIG. 11 (*right*).—Zones of precipitation of horse diphtheria antitoxin (same antiserum as in Fig. 10, but used undiluted as external reactant) with purified diphtheria toxoid (internal reactant) at varying concentrations of toxoid in proportions of 1, $^1/_2$, $^1/_4$. Appearance of the zone is very different from those in Figure 3 because of solubility of the precipitate in excess antibody (33).

COMPLEX PRECIPITATING SYSTEMS.—When 2 or more antigens in a given system cross-react, i.e., when part of the antibody is capable of being precipitated by 1 or the other of the antigens, they cannot react independently of each other. In these circumstances the penetration h of each leading edge is not only a function of the concentration of the particular antigen but also a function of the concentrations of the other cross-reacting antigens (Fig. 12).

If 2 or more antigens cross-react with the given antibody in a gel to give several zones of precipitation, the zone farthest from the interface is due to the antigen which precipitates the least amount of antibody (Fig. 13).

MULTIPLE PRECIPITATING SYSTEMS.—In analyses of naturally occurring mixtures of antigens, multiple precipitating systems will usually be obtained. *In such a system all antigens which do not cross-react will behave independently, i.e., as if each were present alone.*§ Figure 14 shows the behavior of 1 complex and 2 simple systems and that of an artificial mixture containing all 3 systems


E
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gc/2.5 = 1 1 1 ¼ ¼ 1/20 1/20 1/40 1/40 0

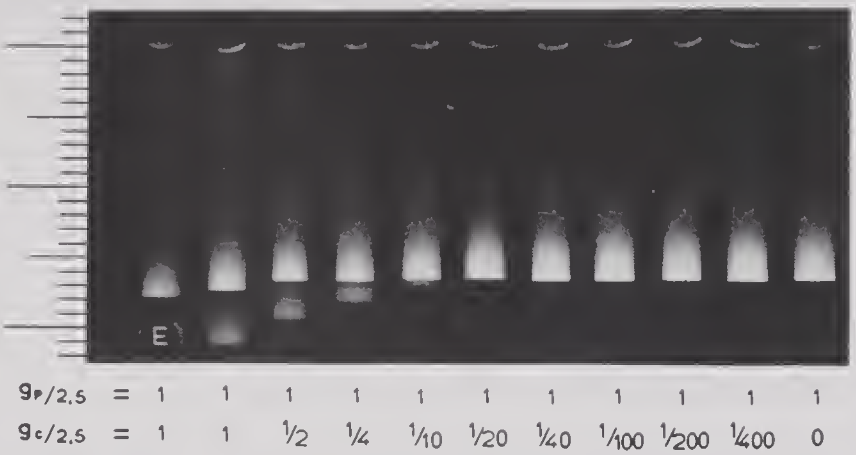
E
gP/2.5 = 1 1 1 1 1 1 1 1 1 1 1
gc/2.5 = 1 1 ½ ¼ 1/10 1/20 1/40 1/100 1/200 1/400 0

FIG. 12 (*above*).—Study of a complex system. Reaction of anti-chicken ovalbumin rabbit serum with mixtures of chicken and duck ovalbumin and with each alone. g_P and g_C are concentrations of chicken and duck ovalbumin, respectively. Penetration of the zone of duck ovalbumin is clearly increased by presence of chicken ovalbumin in 2d and 4th tubes, by comparison with 3d and 5th tubes. Reverse influence is more subtle. *E* under the 1st tube indicates that the antiserum had been absorbed with duck ovalbumin (30, 32).

FIG. 13 (*below*).—Same complex system as Figure 12 (see legend). Constant chicken ovalbumin concentration with decreasing concentrations of duck ovalbumin from left to right. Above a certain concentration of duck ovalbumin, 2 zones may be observed, and penetration of the chicken ovalbumin zone is increased (32).

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§ This is probably not true outside certain limits; a very dense zone of precipitation could constitute an obstacle to diffusion of antigens.

under comparable conditions. It is clear that the picture of the multiple system corresponds closely to that which would be obtained on superimposing the photographs of the isolated systems.

Applications to Immunochemical Analysis.—determination of minimal number of antigens in a system; sensitivity of the technique.—We may conclude from the foregoing discussion, with certain reservations considered later (p. 355), that the number of antigens in a given solution is greater than or equal to the number of leading edges or of maxima of density of precipitate observed in the gel.

As already pointed out, the distance from each leading edge to the interface is nearly proportional to the square foot of time. The ratio of distances between different leading edges, therefore, remains constant with time.

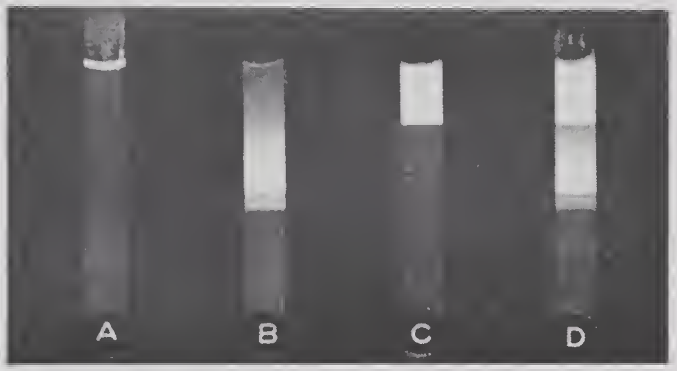

Fig. 14.—Study of an artificial multiple system. Internal reactant consists of the same mixture of antisera in all 4 cells. Antigens are: in *A*, polysaccharide of *E. typhosa;* in *B*, a mixture of chicken and duck ovalbumin; in *C*, horse serum albumin; in *D*, mixture of all 4 antigens, each at the same concentration as in *A*, *B* and *C*. *D* looks like a superposition of the other 3 (30, 32).

The different zones are at first (if precipitation is rapid) superimposed on each other in a very narrow space just below the interface. In the course of time they become progressively more distinct as if they had been magnified, depending on their number, density and the value of $h/\sqrt{t}$ for each zone of precipitation. It remains possible, of course, that 2 leading edges may be so close together that they cannot be distinguished or that a very faint zone may be masked by 1 of high density and greater penetration.

The determination of the number of antigens depends mainly on the concentration of their corresponding antibodies in the serum used and, to a lesser extent, on the concentrations of the antigens themselves.

1. For each antibody there exists a concentration below which precipitation is not visible. The sensitivity in gel is of the same order as in liquid media, or slightly less.

2. The minimum concentration of each antigen that can react to form a visible zone depends on the concentration of antibody and on the antigen-antibody ratio at equivalence. If the concentration of antigen is less than the minimum value required for equivalence, precipitation occurs in the antigen layer. For this reason it is advisable that the concentration be as high as possible. A high concentration does not introduce any complications. If one wishes to be sure that no antigen present in low concentration will escape detection, it is sufficient to use serial dilutions (e.g., 2- or 3-fold) of the antiserum in the gel. Certain zones disappear as the concentration of certain antibodies becomes too low in the higher dilutions of antiserum (see (1)). The remaining zones are easily recognized from 1 tube to the other, and the new zones which appear on dilution of antiserum may be recognized by their lower values of $h/\sqrt{t}$.

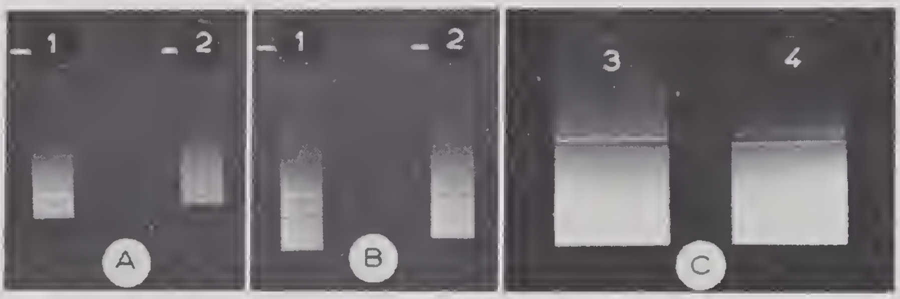

FIG. 15.—Photographs of striations or narrow bands provoked by rapid changes in temperature. Same cells, *1* and *2*, were photographed successively in *A* and *B*. After being allowed to develop for several days at 22 C, the cells were placed, one at 37 C (*1:* increased density of striation), the other at 4 C (*2:* reduced density of striation). *A* was taken at the moment the 2 cells were replaced at 22 C. This change caused, at the level of the boundary, a new striation, seen in *B* taken several days later. *C*, photograph at greater magnification of 2 other cells: *3*, placed at 37 C for about 15 min, then returned to 22 C; *4*, placed for 15 min at 4 C, then returned to 22 C (30, 32).

Sources of Error.—1. *Nonspecific precipitation:* The problem is the same here as in reactions in liquid media and is handled similarly. Normal serum controls described previously should be set up in the gel (see pp. 301 ff.).

2. *Artefacts due to changes in temperature:* A rapid change in temperature, even small, can provoke the formation of a narrow band whose density is different from that of the surrounding precipitate[‖] (Fig. 15). These artefacts appear at the level of the leading edge at the time of the temperature change and remain fixed at this level while the zone continues to migrate. They are

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[‖] It is possible that the appearance of the tubes observed by Brown (4), in which the reaction was allowed to proceed in the refrigerator, was due to artefacts of this kind.

due to a sharp change in the progress of the diffusion and demonstrate that the diffusion of antibody is not 0, even though, as a simplifying assumption, it has been neglected. The appearance of these artefacts is rather characteristic; moreover, their lack of mobility contrasts sharply with the regular progression of the true boundary.

3. *The Liesegang phenomenon* should be mentioned here in view of the role attributed to it in the past. With inorganic substances such as $K_2Cr_2O_7$ and $AgNO_3$, it consists essentially of a discontinuous precipitation in the form of separate bands whose thickness and spacing are periodic (11, 39). Such successive bands remain immobile (39); in our studies we have never observed this phenomenon in immunochemical reactants.

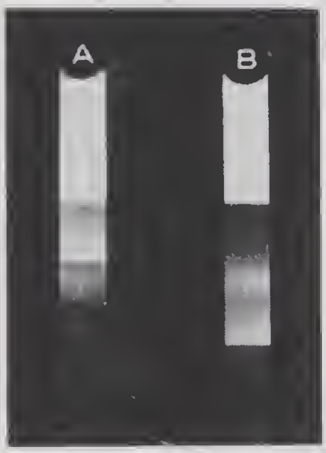

FIG. 16.—Photograph of 2 cells each containing 3 diffusing antigens in the same gel. The only difference between them is that the concentration of 1 antigen, ovalbumin, is much higher in *B* than in *A*. Penetration of the zone of this antigen is therefore greatly increased in *B* as compared with *A* (30, 32).

IDENTIFICATION OF COMPONENTS.—The problem of identification is the following: A given solution *M*, containing several antigens, reacts with an anti-*M* serum A_M to give several zones. One wishes to know which of these zones are produced by antigens common to *M* and to another solution *L*, suspected to contain 1 or more of them.

When the antigens in *M* and *L* capable of reacting with A_M are few in number, e.g., 3 or 4 in *M* and 1 or 2 in *L*, one can, in favorable circumstances, hazard a guess as to the corresponding zones by comparing tubes in which *M* and *L* react separately with A_M. Any identical antigen present in both tubes will give a zone of the same maximum of density of precipitate, a property which is independent of concentration.

The independence of reaction of different antigens suggests several procedures for identifying them with certainty, for if one changes the concentration of only 1 antigen or of its antibody without affecting the concentration of the other reactants (antigens or antibodies), only 1 zone will be affected (this would not necessarily be true in the special rare case in which one changed the concentration of 1 of 2 cross-reacting antigens in *M*).

1. The 1st consists in comparing 2 tubes, 1 of which contains

the antigens of L in higher concentration than the other (Fig. 16). In practice one prepares a dilution of M in L and a similar dilution of M in any convenient diluent (normal saline, or serum from unimmunized animals). Each dilution is allowed to react separately with A_M in tubes. If comparison reveals that certain zones in $M + L$ show a higher penetration than in tube M, these zones are caused by antigens present in L. The higher the concentrations in solutions L of the antigens common to both M and L, the more successful will this procedure be, provided the zones due to the reaction M–A_M are not too numerous to make comparison between the tubes difficult.

2. The author usually prefers to use another procedure which involves the more or less complete absorption of the antibodies to L which are present in A_M. Three situations are possible:

a) Only a single antigen is common to M and L, which allows the determination of the equivalence ratio of L–A_M and the absorption of A_M by a slight excess of antigen L.

b) Several antigens are common to M and L, and it is both useful and easy to absorb A_M completely with L. One carries out this absorption by mixing A_M with a quantity of L such that the supernatant no longer precipitates with L. By comparison of tubes in which M reacts respectively with this supernatant and with unabsorbed A_M (similarly diluted), the zones common to M and L may be identified since they disappear after absorption; the zones which remain correspond to antigens of M which are not present in L (see example in (32)).

c) Several antigens are common to M and L, but only those found in L in an appreciable concentration are of particular interest. This is the case when M is separated into fractions, L_1, L_2, etc., each containing all the antigens of M at least in trace quantities. A_M is absorbed by a given quantity of a given fraction of L. All the zones which disappear after absorption are due to antigens present in L, but any zones which may remain can be due to antigens present in L in low concentration.

The procedure involving absorption, although somewhat longer than procedure 1, is more certain. In contrast to procedure 1, and also to the supplementary means offered by the technique of 2-dimensional diffusion (coalescence of zones, see p. 370), a great number of zones does not affect the ease of reading the tubes (Fig. 17).

In the foregoing discussion we have considered the most general case, namely, that of identifying zones due to antigens of identical specificity present in both L and M using an antiserum homologous for M. Different results would, of course, be obtained if certain of the antigens of *L* and *M* cross-reacted in anti-*M* serum. The investigation of complex systems suggests that in the case of a cross-reacting antigen, procedure 1, i.e., increase of antigen concentration, could result in (*a*) a slight increase in the penetration of the corresponding cross-reacting zone of *M* with a diminution in the maximum density of precipitate, and (*b*) the appearance of a new zone which migrates more rapidly than the homologous zone (Fig. 13, tubes right to left except the 1st tube on the left).

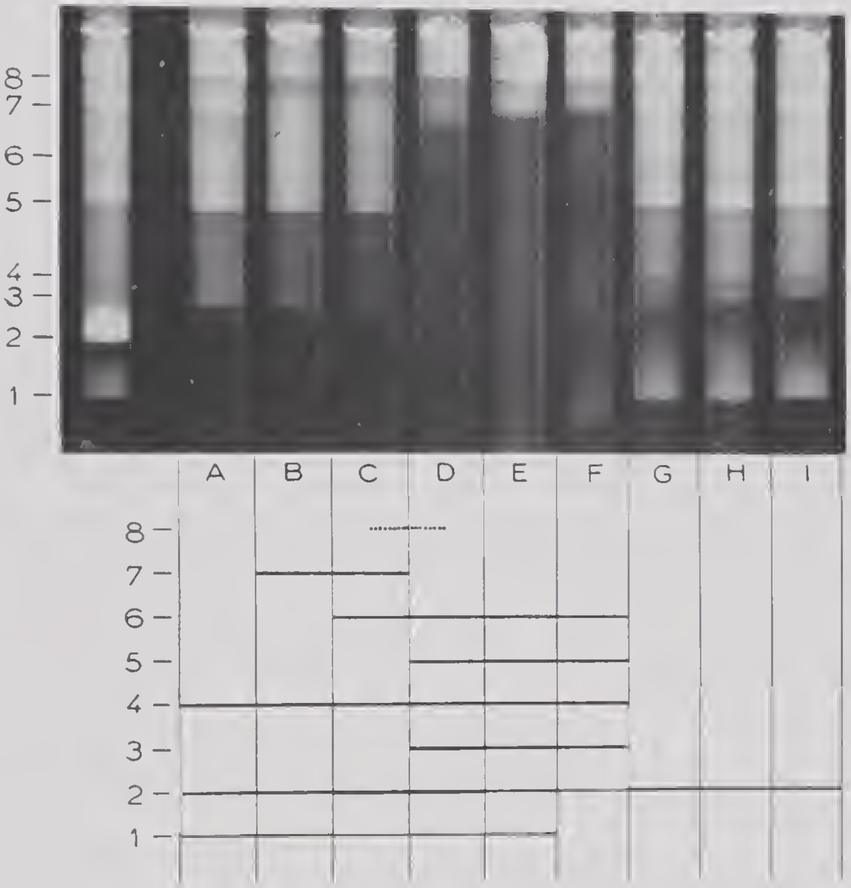

FIG. 17.—Identification by means of absorption. A human serum globulin fraction has been separated into 9 subfractions by salting out: *A*, *B*, *C*, etc. In the 1st tube: reaction of the original fraction of antigens with homologous antiserum (8 zones). In the 9 following tubes: reactions of the original fraction with the same immune serum absorbed by a constant quantity of each of the 9 fractions, respectively; every zone which disappeared (in comparison with the 1st tube) corresponds to an antigen present in the subfraction under consideration. This permits establishment of a scheme showing distribution of 7 antigens in the 9 fractions. The low penetration of zone 8 shows that concentration of antigen 8 is weak. When larger quantities of fraction *C* and *D* were used in the absorption, zone *8* was modified. By varying the amount used for absorption, one can construct a curve showing relative concentrations of each antigen in the various fractions (33).

Procedure 2 (absorption) would not show the complete disappearance of the corresponding zone, but only a diminution in its maximum density of precipitate and an increase in its penetration. Both effects become more marked as the absorption becomes more complete, in other words, as the reaction approaches that of the homologous antigen (Fig. 13, compare 1st and last tubes). When there are more antigens reacting, the analysis becomes more difficult.

SIMPLE DIFFUSION IN 2 DIMENSIONS

Simple diffusion in 2 dimensions, i.e., diffusion in a layer of agar rather than in a tube, is little used and has not been studied quantitatively. A technique has been briefly described by Ouchterlony (19, 20, 26), who used diphtheria toxin diffusing from a "penicillin cup" into a layer of agar containing antitoxin. One or several zones of precipitation concentric at the source of the diffusing reactant appeared in the gel after a variable length of time. Reasons for the delay are discussed on page 343.

Although qualitatively analogous, it is incorrect to reason that the quantitative relationships which describe diffusion in 1 dimension apply here along each line which radiates from the center of the reservoir. Actually, in the tube, when the leading edge of a zone is at a distance h from the interface, the molecules which have crossed the interface redistribute themselves, unequally according to the laws of diffusion, in a volume proportional to h. In two-dimensional diffusion, they are redistributed in a volume proportional to πh^2.

Petrie observed the coalescence of haloes which developed around neighboring bacterial colonies (34). It will be seen later how the coalescence of zones of precipitation may serve as a means of identification in double diffusion. No one appears to have used the coalescence of zones produced by the same antigen, diffusing from 2 different sources in a gel containing immune serum, for purposes of identification.

DOUBLE DIFFUSION

In what will be termed double diffusion, 2 reactants diffuse toward each other through a gel which contained neither reactant at the beginning of the experiment. The possibility of double diffusion in 1 dimension (in tubes) as a method of immunochemical analysis was suggested early by the author (27). However, no precise technique has been published.*

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* During translation of this chapter, a paper by Pope *et al.* (35a) appeared in which the following technique for double diffusion in tubes of gel was used. A layer of antiserum incorporated in gelatin is placed in the bottom of each tube. This is covered with a layer of gelatin, followed by a 3d layer containing the antigen in gelatin. The diffusion is carried out in the cold. The authors credit the technique to unpublished work by Oakley.

DOUBLE DIFFUSION IN 2 DIMENSIONS

Several procedures for double diffusion in 2 dimensions (in layers of agar) have been described by Ouchterlony and by Elek. In their early papers (19, 8) both described techniques designed to study toxin production by living cultures, in particular *C. diphtheriae* (see p. 374). Later they described other techniques applicable to the analysis of solutions of antigens (22, 25, 8).

In the present discussion, "source of diffusion" means the boundary which separates the reservoir containing a solution of the diffusing reactant from the gel into which the diffusion takes place. Two cases will be considered in which the sources of diffusion are rectilinear: (1) diffusion of antigen and antibody from parallel sources which face each other, forming opposite sides of a rectangle (see Fig. 20, *A*); (2) diffusion of antigen and antibody from sources perpendicular to each other (see Fig. 20, *B* and *C*).

We will limit the discussion to these 2 cases, although any number of other techniques could be imagined, varying according to the form, number and position of the sources of reactants. What is true for the 1st of these 2 techniques is applicable *mutatis mutandis* to the 2d.

DIFFUSION FROM 2 OPPOSITE PARALLEL SOURCES.—TECHNIQUE.—This technique was described by Ouchterlony (22). Except with regard to its concentration, the agar used in the various techniques of double diffusion may be prepared as described for simple diffusion in tubes. Ouchterlony suggests that the agar, at a concentration of 60 g/l, be clarified by precipitation with calcium chloride, filtered through glass wool and, after solidification, cut into small cubes and washed with running tap water for 3 days, then with distilled water, and finally diluted while hot to $^1/_4$ its initial concentration. This dilution is made after adding a quantity of NaCl such that its final concentration is 0.8 per cent. The final concentration of agar is therefore appreciably less than 1.5 per cent.

The agar is poured in 2 successive layers into a Petri dish of 10 cm diameter. One first pours a thin layer which is allowed to solidify. Two metallic molds, 15 × 35 or 10 × 10 mm, covered with a very thin layer of paraffin, are then placed parallel to each other at opposing sides of a rectangle and 20 mm apart on the 1st layer of agar (see Fig. 20, *A*). A 2d layer of agar about 3 mm deep is then poured around these molds. After the agar has solidified, the 2 molds are very carefully removed. The reservoirs formed by this procedure are then filled with the reactants. "Every 12th or 24th hour, depending on how rapidly the diffusion into the . . . . agar takes place," the reservoirs are refilled. Ouchterlony carries out the reaction at 37 C.‡ The plates can be examined in oblique light under a microscope giving 10-fold magnification. Eventual quantitative measurements are made by means of a graduated mechanical stage and a cross-hair eye-piece. The plates can be photographed.

THEORY AND RESULTS.—The same simplifying hypotheses presented for simple diffusion are applicable here, with the obvious exception that the concentration of neither reactant remains constant in the gel. Moreover, as Ouchterlony has pointed out (23, 26), the diffusion of each reactant can, without significant error, be considered as diffusion in 1 dimension at least along the line which joins the center of each source, neglecting the fact that the depth of the reservoir is less than the thickness of the agar layer. The concentration of each of the reactants at any instant and at any point

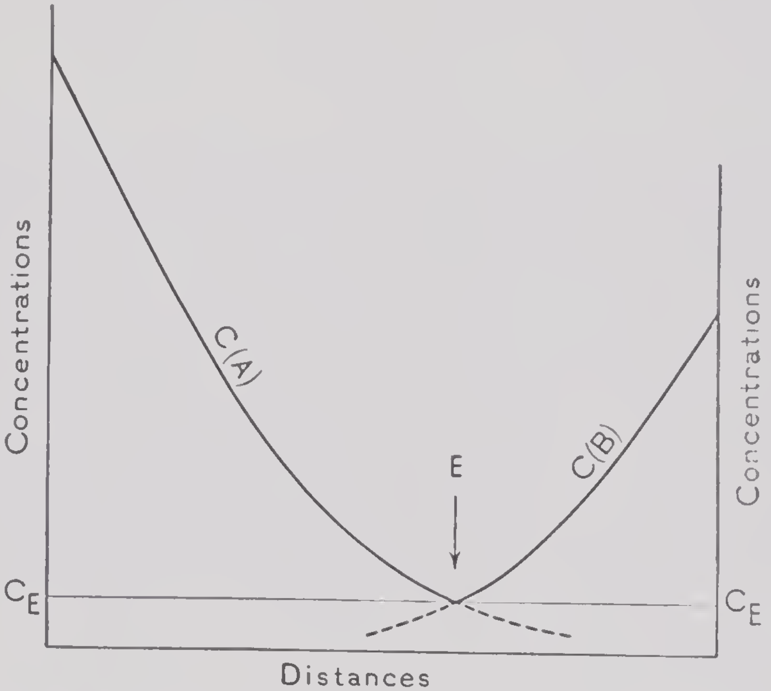

FIG. 18.—Diagram showing double diffusion at given time *t*. Concentrations *C*(*A*) and *C*(*B*) of the 2 reactants *A* and *B* are expressed in equivalent units. Crossing of the 2 curves indicates the point of equivalence E. C_E is the concentration of the 2 reactants at this point. Curves *C*(*A*) and *C*(*B*) were drawn following the theoretical laws of diffusion, which is only justifiable as long as C_E can be considered negligible with respect to initial concentrations; the dash-line portion of the 2 curves therefore has no precise significance. It is evident that C_E increases in the course of time.

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‡ For convenience, the reactions illustrated in this chapter were carried out at about 18 C using square reservoirs 10 × 10 mm in Petri dishes of 55 or 75 mm diameter. Theoretically, a dish of small diameter favors the phenomenon of reflection of the diffusing molecules by the walls of the vessel. This is sometimes manifested by deviation at the extremities of zones of precipitation sufficiently extended as to reach nearly to the walls of the dish.

in the gel, where the combination with the other reactant has not yet taken place, should theoretically be described by either equation (2) or equation (3), which relate the concentration of the reactant (considered constant at the interface between solution and gel) and its coefficient of diffusion (Fig. 18). The precipitate will appear at those points and at that time when the 2 following conditions are realized: (1) when the concentrations of the reactants reach a threshold level sufficient to allow the formation of a visible precipitate, and (2) when the ratio of the concentrations of the 2 reactants favors the formation of insoluble complexes.

If the initial concentrations of the 2 reactants are in equivalence ratio, the theoretical equations predict that the point where the 2 reactants will meet at equivalent concentrations (defined here as "point of equivalence") is situated at distances from the sources of diffusion which are proportional to the square root of the coefficients of diffusion of the reactants (23, 26), and that in the course of time the concentrations of reactants will remain in the same ratio at the point where they first met. As a result, the zone of precipitation, or at least its maximal density, will remain stationary with time.

Ouchterlony, using this method, has determined the ratio of the coefficient of diffusion of horse antitoxin to that of diphtheria toxin. The ratio found was somewhat less than the value determined by free diffusion in liquid media. Ouchterlony attributes this difference to the influence of the gel. In this particular case (where initial concentrations are in equivalence ratio) the precipitation zone appears as a thin immobile sheet of precipitate whose density increases as the 2 reactants continue to reach it.§

In most cases, antigen and antibody will not be present in equivalent initial concentrations, and the point at which precipitation first appears will be displaced in a direction away from the source of whichever reactant is present in excess. Furthermore, the point of equivalence will not remain stationary but will migrate away from the source of the reactant which is in excess.

If the reactant in excess is the antigen, both edges of the zone of precipitation will usually migrate, since the specific precipitate can in most cases be dissolved by the strong concentration of excess antigen diffusing into the zone. The maximum density of the zone will increase during its migration toward the antibody source.

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§ It may be noted that when the initial concentrations of the reactants are in equivalence ratio and the sources of diffusion are symmetrical, the sensitivity of the reaction is limited by the total quantity of reactants and by the duration of the experiment and *not* by their initial concentrations. One may therefore predict that under suitable conditions a greater sensitivity may be reached than in the ordinary ring test. The sensitivity will be greatest when the distance which separates the reservoirs is small and when their volume is large in comparison with that of the gel. For these reasons, it is to be expected that high sensitivity can be obtained even more readily in tubes.

If the reactant in excess is antibody, we would expect the edge of the zone of precipitation nearest the source of antibody to remain stationary in a precipitin system. Therefore only the edge facing the antigen source will migrate and the zone will continue to broaden. This is not true for systems of the flocculation type in which the specific precipitate is soluble in excess antibody (Fig. 19).

It is reasonable to assume that for any given antigen and antibody the concentrations required to give just visible maximum density of precipitate will always be the same under similar conditions.[‖] The time required for the concentrations of antigen and antibody to attain the threshold value required for precipitation at a point of the gel will be proportional to the square of the distance between the sources according to the laws of diffusion. This relationship would always be true for the time of appearance of the zone of precipitation if the time for formation of precipitate could be neglected. Furthermore, the time for the appearance of precipitation is longer when the initial concentrations are weaker.

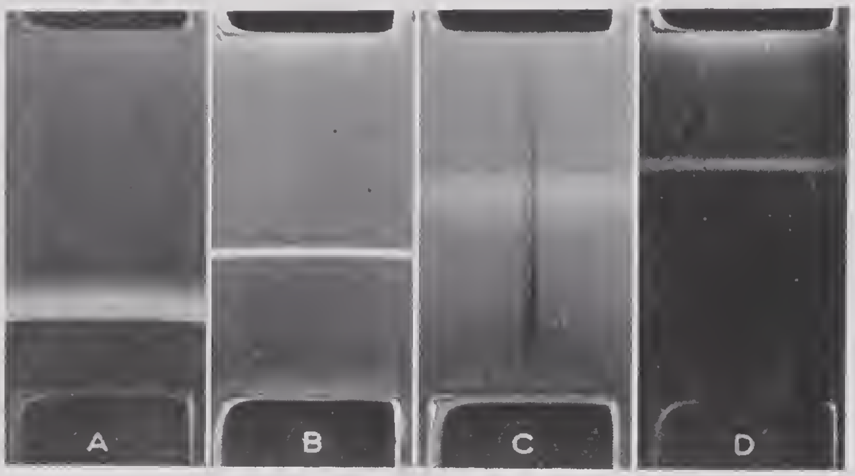

FIG. 19.—Photograph illustrating behavior of simple systems using double diffusion in agar plates (Ouchterlony's technique). The 2 square reservoirs were placed face to face at a distance of 20 mm. The lower contained the antiserum and the upper, the antigen. *A*, *B*, *C*: reaction of ovalbumin, at 3 different initial concentrations, with homologous rabbit antiserum (approximately 1 mg specifically precipitable N/cc) at constant initial concentration. *A*, large excess antigen; *B*, antigen close to equivalence concentration; *C*, large excess antibody; *D*, reaction of purified diphtheria toxoid with excess horse antitoxin (same reactants as in Figs. 10 and 11) (33).

Theoretical Basis for Determination of Number of Reacting Antigens.—The reasoning which showed that each zone of precipitation corresponds to a single antigen-antibody reaction in the case of

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[‖] Ouchterlony calculated the critical concentrations for the diphtheria toxin-horse antitoxin system by trial and error (23, 24).

simple diffusion is not valid in the case of double diffusion. Ouchterlony does not exclude the possibility "that multiple streaks may be formed by a single antigen which reacts at different points in the diffusion medium with various antibodies with different diffusion rates and reactivity" (22). A priori, this is not likely, for it seems logical to suppose that for a single precipitating system the point of equivalence divides the gel into 2 regions: that of excess of antigen, where all the molecules of antibody are saturated by antigen, and that of excess of antibody, where all the molecules of antigen are saturated by antibody. It is to be expected that at the point of equivalence which separates the 2 regions both antigen and antibody are completely immobilized by their mutual combination and precipitation. For this reason neither can pass into the region containing the other.* Only experimental study of a number of isolated known precipitating systems can resolve this question, as was done in the case of simple diffusion. The simple systems studied to prepare the illustrations for this chapter have each given a single zone of precipitation, with certain exceptions, the causes of error for which are known and are discussed later (p. 372).

The independence of zones of precipitation due to distinct antigens which has been established for simple diffusion (p. 353) is obviously not restricted to any 1 type of diffusion and therefore should be applicable to the present case. Therefore, the zones due to distinct antigen in a multiple system should appear, after a variable delay, at different points in the medium, as observed by Ouchterlony, and each of these zones should remain immobile or be displaced toward the source of antigen or of antibody, depending on which is in excess.

Identification can be carried out by the absorption technique discussed for simple diffusion.

Diffusion from 2 Perpendicular Sources.—techniques.—Two techniques have been described.

Ouchterlony (22) prepares his agar plates as already described (p. 360), either by placing the same molds, 15 × 35 mm, at right angles or by using 3 square molds of 10 mm on a side† and mounted together in fixed position (Fig. 20, *B* and *C*); one can use all 3 reservoirs (see p. 370) or only 2 of them by filling the 3d with agar.

Elek (8) chooses as his reservoirs strips of filter paper. For example, a strip of filter paper 10 × 60 mm, containing exactly 0.1 cc of diphtheria antitoxin (4,000 units/cc), is immersed in the agar while it is still liquid. Once the agar has solidified a similar strip containing 0.1 cc of concentrated antigen solution (e.g., diphtheria toxin, 4,000 Lf units/cc) is placed on the surface of the gel, forming a right angle with the preceding strip. After a convenient time, the plates may be placed on photographic paper and contact prints made.

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* Insofar as this reasoning is valid here, it is also valid for simple diffusion, where one comes to the same conclusion by other means.

† Each metal mold is pierced by a central hole to avoid sucking up agar when the assembly is withdrawn.

THEORETICAL ANALYSIS.‡ To understand what takes place in the gel as a whole, let us assume that at a given distance the concentration of each reactant is equal at all points lying along a line parallel to its source of diffusion. This assumption would only be fully justified if the source were long and if one were to neglect points lying near its extremities. It is considered sufficient, however, to describe the distribution of concentrations of the reactant along a single perpendicular to its source to understand what happens along other perpendiculars. Furthermore, the diffusion along each perpendicular may be considered as simple diffusion.

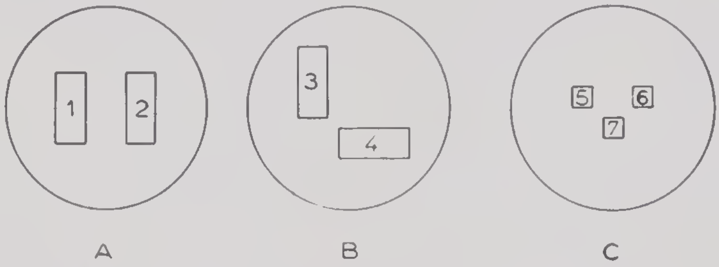

FIG. 20.—Forms and arrangements of reservoirs. Dimensions of molds were: for *1*, *2*, *3* and *4*, 15 × 35 mm; for *5*, *6* and *7*, 10 × 10 mm; distance from *A* to *B* and from *E* to *F*, 20 mm. Different variations of form, of number and of arrangement of reservoirs can naturally be imagined (after Ouchterlony (22)).

Let us finally assume that the concentration of each reactant remains constant in the neighborhood of its source so that we may apply either equation (2) or (3). In practice, this condition is far from true.

The curves shown in Figure 21 were constructed after making the above simplifying assumptions and neglecting all effects due to mutual combination and precipitation of the reactants. The sources of diffusion are drawn contiguous at their extremities in Figure 21.

Under the ideal conditions so defined, the specific precipitation of the reactants during the course of their diffusion will resemble to a certain extent what we have seen in simple diffusion and will resemble even more closely double diffusion from 2 parallel sources.

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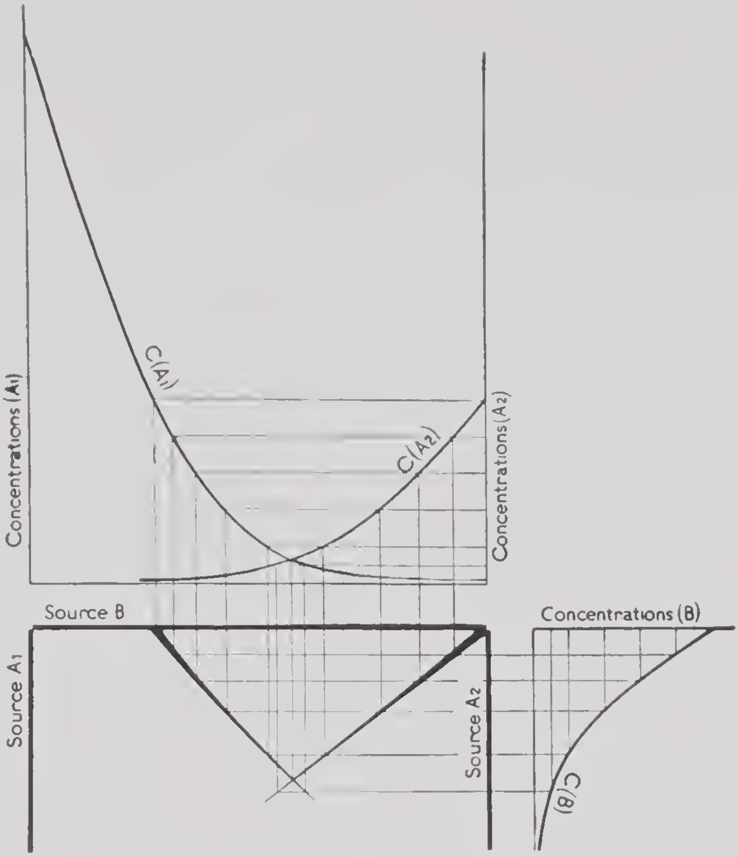
‡ The theoretical aspect of this type of diffusion has been discussed by Elek in a somewhat different manner (8).


C(A1)
C(A2)
Concentrations (A1)
Concentrations (A2)
Source B
Concentrations (B)
Source A1
Source A2
C(B)

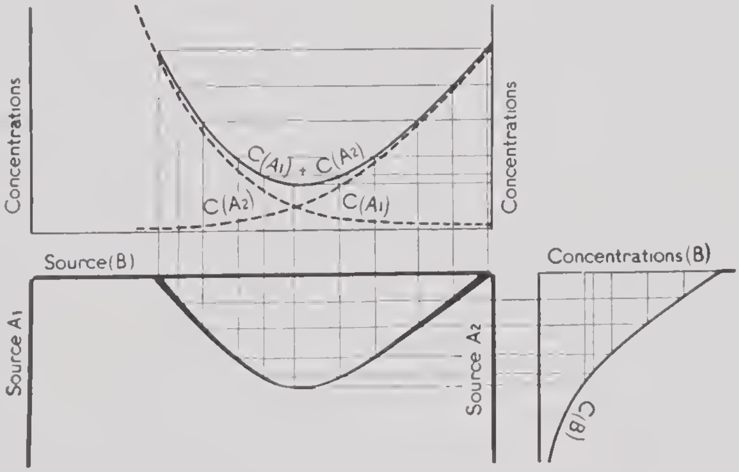

Concentrations
C(A1) + C(A2)
C(A2)
C(A1)
Concentrations
Source(B)
Concentrations (B)
Source A1
Source A2
C(B)

FIG. 21 (*above*).—Schematic representation of double diffusion from 2 perpendicular sources at a given time t. Consider B as antibody, and A_1 and A_2 as 2 antigens with distinct specificity but the same diffusion coefficient. One assumes that antibodies anti-A_1 and anti-A_2 are at the same initial concentration and have the same coefficient of diffusion, which is lower than that of A_1 and A_2; their concentrations are therefore represented by the same curve $C(B)$.

Curves of the concentrations conform to the theoretical laws of diffusion in 1 dimension, with the hypothesis that diffusion of each reactant along each perpendicular to its source conforms to these laws. Because of the conventional choice of equivalent units of concentration of antigen and antibody (as for graphs in Figs. 1 and 18), each line of equivalence describes the locus of points where concentrations of antigen and antibody are equal. To give an idea of the variable density of precipitate, these loci are represented by tracings whose

Let us consider the diffusion of 1 reactant, A, along any perpendicular to its source. We may assume that at all points along such a perpendicular the concentration of the other reactant, B, will be equal at any given time t but will increase with time. If we now consider perpendiculars brought closer and closer to the source of reactant B, then *at the limit* the concentration of reactant B not only will be equal along the perpendicular at time t but will remain constant with time; these are precisely the theoretical conditions encountered in simple diffusion, and the conclusions which follow from the theoretical study of simple diffusion should apply to this limiting case as well. In particular, the point of equivalence in the gel is very close to the intersection of the 2 sources at time $t = 0 + \epsilon$ no matter what the initial ratio of antigen and antibody concentrations.

Aside from the limiting case, one may apply what has been said regarding double diffusion from 2 parallel sources to the condition in which the sources are perpendicular by simply considering for each point in the gel its perpendicular distances to the sources of diffusion.

When both reactants have equivalent initial concentrations (Fig. 21; reactants A_2 and B) the perpendicular distances from the points of equivalence to each of the 2 sources of diffusion will remain in constant ratio, the value of which will be the same as that found for double diffusion from 2 parallel sources. In this particular instance, the geometric locus of the equivalence points will be a segment of a straight line originating at the intersection of the 2 sources of diffusion. The angles formed between this line and the sources of diffusion will remain constant with time. The ratio of the sines of these angles will have the same value as would the ratio of the distances from the point of equivalence to the sources of diffusion in the case in which the same reactants are diffusing from

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thickness is roughly proportional to concentrations of the reactants at each point.

The initial concentrations of A_2 and anti-A_2 (B) are in the ratio of equivalence (equal on the graph). The line of equivalence is straight and passes through the intersection of the sources. This straight line does not bisect the angle between the sources, because the coefficients of diffusion are different for A_2 and anti-A_2 (B).

Form and position of the line of equivalence of A_1 (different from those of A_2) are due to the excess initial concentration of A_2 (3 times that of A_1) with respect to anti-A_2 (B); 1 of the 2 reactants being in excess, the line of equivalence passes through the intersection of the sources only at the time $0 + \epsilon$ (33).

FIG. 22 (*below*).—Conditions are the same as in Figure 21, with 1 exception: identical antigens, instead of 2 different ones, diffuse from sources A_1 and A_2 (at same concentrations as in Fig. 21). Locus of the points of equivalence is therefore drawn here by taking as concentration of antigen the sum of the concentrations $C(A_1)$ and $C(A_2)$ at each point. This explains the coalescence of the lines of equivalence of A_1 and A_2 (33).

parallel sources;[§] in other words, the same value as the ratio of the square roots of the diffusion coefficients.

The maximum of the density of precipitate for each perpendicular from 1 or the other source of diffusion will be at a point close to the point of equivalence. This maximum density will increase with time at any given point, and at any given time will become less and less as one considers points in the gel farther and farther from the sources of diffusion until finally no precipitate is visible. In analogy to what we have seen for diffusion from 2 parallel sources, the point at which the zone of precipitate is no longer visible will be displaced in such a manner that the perpendicular distances from this point to the sources of diffusion would be proportional to the square root of time if the time of formation of the precipitate could be neglected.

When the reactants are not present in equivalent concentrations (Fig. 21; reactants A_1 in excess and B) the zone of precipitation will be displaced in a direction away from the source of diffusion of the reactant that is present in excess. The appearance of the zone of precipitation will differ from that of the preceding case as would be expected from what has been said regarding diffusion from 2 parallel sources (see p. 362 and Fig. 19). Naturally, here as in the preceding case the density of precipitate will diminish at points farther and farther away from the sources of diffusion.

PRACTICAL CONSIDERATIONS.—In practice, experimental conditions for double diffusion at right angles deviate from the ideal ones discussed above according to the particular technique chosen. The most important differences are: (1) The 2 sources cannot be exactly contiguous as in Figure 21. (2) The concentration of the reactants will not remain constant at the sources of diffusion if the volume of the reservoirs into which they are placed is small in comparison with the volume of gel into which they are diffusing.[‖] (3) Of necessity, the depth of the reservoirs must be less than the thickness of the agar.

Ouchterlony's technique deviates from the ideal particularly with respect to points (1) and (2). The deviation is less serious with respect to point (3) since the lower layer of agar is usually thinner than the upper layer. Because of the appreciable distance that antigen and antibody must diffuse (even from the points closest together between the 2 sources of diffusion), an appreciable time will elapse before concentrations are built up sufficiently high to result in visible precipitation. If the initial concentrations of the reactants are low, 1 or more days may be necessary before the appearance of a precipitate. In a multiple system this period will, of course, be different for each of the different zones of precipitation. Since the reservoirs do not touch each other, the zone of precipitation will first appear and remain most dense in the space which separates them and will form an obtuse angle since we must now consider diffusion from adjacent sides of the same reservoirs.

[§] This reasoning finds experimental confirmations in results given by Ouchterlony (22); the distances found are, respectively, 8.5 and 20 − 8.5 = 11.5, the angles 37° and 90° − 37° = 53°; the ratio of the distances is thus 0.74 and of the sines 0.75.

[‖] Ouchterlony (23) notes that the concentration of reactants in his reservoirs decreases about 25 per cent within 12 hr.

The periodic refilling of the reservoirs required by Ouchterlony's original technique compensates to some extent for the fall in concentration of the reactants within the reservoirs. One modification suggested by Ouchterlony eliminates the necessity for refilling the reservoirs by sealing the Petri dishes with Plasticene to prevent evaporation. Although this modification leads to somewhat different results and deviates from ideal conditions, it does not appear to diminish considerably the value of the qualitative findings. For reasons to be discussed, it seems to us preferable to avoid refillings in qualitative experiments.

Elek's technique deviates from ideal conditions particularly with regard to points (2) and (3). The reservoirs are particularly small in this case since their content is "exactly 0.1 ml." for a filter paper strip 10 × 60 mm. Presumably it is for this reason that Elek uses antitoxin and toxoid of very high titer (4,000 units/cc). For the same reason, in his analyses of human serum Elek found it necessary to dry successive quantities of the immune serum in 3 strips of filter paper which were then superimposed to serve as the reservoir of antibody.*

The very rapid fall in concentration in reservoirs of such small volume probably explains also the stationary "planes of flocculation" described by Elek, an observation quite contrary to the findings by Ouchterlony's technique, particularly when the antigens are as highly concentrated as certain of the constituents present in serum. In Elek's procedure the depth of the reservoirs containing the reactants is very small compared with the thickness of the agar. Moreover, the filter paper strips are not held in the same plane, so that they are separated by a thin layer of agar near the vertex of the angle which they form. The geometric locus of the points of equivalence in this region will be a plane parallel to the strips and will, of course, pass between them. In regions at a distance from the sources of diffusion this locus will become a plane perpendicular to the gel; it thus undergoes a rotation and twisting in the neighborhood of the angle formed by the sources (8).

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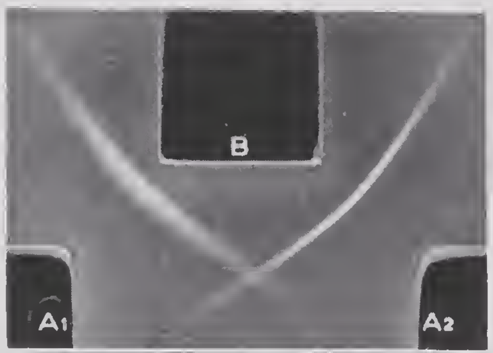
* The number of zones of precipitation found for human serum by this procedure was not indicated. When the strip of filter paper was placed vertically and only partially submerged in the agar so that the protruding edge could be moistened each day with fresh immune serum, 5 zones were obtained, a number which is a good deal less than that obtained by other methods ((28) and Fig. 17). Perhaps this procedure could be still further perfected by dipping the protruding edge of filter paper in a large volume of reactant so as to avoid fluctuations in concentration.

Diffusion of Same Reactant from 2 Parallel Sources; Coalescence of Zones.—Diffusion in 2 dimensions allows one to place 2 or more sources of diffusing reactants in the same gel (22, 8). We will limit ourselves to the simplest case, that in which 2 sources of reactants are placed parallel to each other and perpendicular to a 3d. For example, 2 different solutions containing antigens in common could be placed at the parallel sources and antiserum at the perpendicular source.



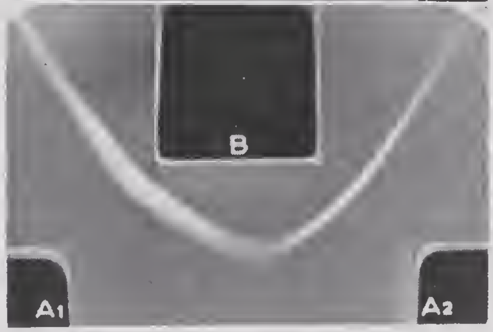


Fig. 23 (*above*).—Photograph of zones of precipitation of 2 diffusing antigens (Ouchterlony's technique) under conditions as close as possible to the theoretical conditions of Figure 21. The 2 antigens were ovalbumin (A_2) whose initial concentration is in equivalence with that of the antibody, and human serum albumin (A_1) at 3 times the concentration of the ovalbumin. The immune serum (B) is a mixture such that the same volume precipitates at equivalence with equal quantities (in terms of protein N) of the 2 albumins (33).

Fig. 24 (*below*).—Same conditions as for Figure 23 except that A_1 contains ovalbumin at an initial concentration 3 times its concentration in A_2 and equal to that of human serum albumin used in Figure 23. Note coalescence of zones of precipitation. The experimental conditions approximate as close as possible to the theoretical conditions of Figure 22 (33).

The graphs shown in Figures 21 and 22 describe the expected behavior using 2 distinct antigens (Fig. 21) and identical antigens (Fig. 22) at the 2 parallel sources. If the 2 antigens are of entirely different specificity, each will react with its respective antibody independently of the other† (p. 353 and Fig. 14). The geometric loci of their respective points of equivalence will cross without disturbing each other, as observed by Ouchterlony and Elek (Figs. 21 and 23).

If, under similar conditions, the same antigen is placed at both parallel sources, then at each point in the gel the concentration of this antigen will be the sum of the amounts which have diffused from each source. The resulting loci of the points of equivalence will therefore be displaced and bent toward the opposing source. The displacement of the locus from 1 source will increase as the other source is approached, and the effect of the concentration of antigen diffusing from the opposite source becomes more and more pronounced. Instead of crossing, the loci of the points of equivalence will eventually join and form an arc‡ (Figs. 22 and 24).

As Ouchterlony has pointed out: (1) if the concentration of a particular antigen is the same at both sources, the coalescing zones will be arranged symmetrically, and (2) an asymmetrical arrangement of the zones indicates that the highest concentration of antigen is present in the reservoir situated farthest from the point of coalescence.

Partial coalescence: When antigens which cross-react are placed at the parallel sources of diffusion and an antibody homologous to 1 but not to the other is placed at the perpendicular source, Ouchterlony has observed in the author's laboratory a partial coalescence of the type shown in Figure 25.

APPLICATION TO IMMUNOCHEMICAL ANALYSIS.—*Determination of Minimum Number of Antigens in a System; Sensitivity of Techniques of Double Diffusion.*—We have considered the theoretical basis for determination of the number of reacting antigens by means of double diffusion (p. 363). In practice, the ease of enumeration will depend on whether it is of the flocculating type, which will usually give sharp zones when the reactant in excess is antibody, or whether it is of the precipitin type, which will give sharp zones only when the initial concentrations of antigen and antibody are close to their equivalence ratio. It should be recalled that at low initial concentrations of antigen and antibody the appearance of the zone of precipitation will be delayed and that the zone given by an antigen whose initial concentration is in great excess over that of antibody may reach the antibody source within a few days. For this reason the plate should be examined at intervals so that no zone escapes detection.

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† The segregation of zones is proof of this independence, but this cannot be used as evidence either for or against the theory that the 2d phase of precipitation is specific, as Elek has claimed (8).

‡ Petrie (34) noted that halos surrounding neighboring colonies of meningococci formed in antiserum "have coalesced." Ouchterlony (19) speaks of "interference," Elek of "looping" and others of "fusion." It is clear that all of these terms pertain to the phenomenon observed by Petrie.

Under ideal conditions for diffusion from perpendicular sources (when sources are contiguous and concentrations of reactants remain constant at the sources), one might expect the sensitivity to be of the same order of magnitude as that pertaining to simple diffusion (at least in the regions closest to the sources, see p. 367). In practice, the exact limit is hard to define because so many variables must be considered. Thus in Ouchterlony's technique the sources of diffusion are not contiguous, time plays an important role and the densities of the zones which are faint at 1st later increase in a manner which depends on the respective concentrations of the antigen and antibody. Moreover, since the rate at which the concentration of the reactants diminishes depends on their volume, the limit of sensitivity in double diffusion will depend on the size of the reservoirs. As we have already seen, this applies especially to Elek's technique.

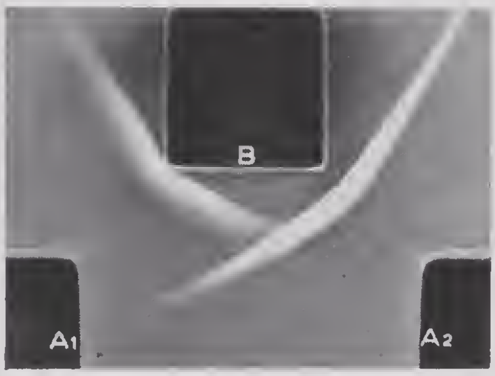

FIG. 25.—Cross-reaction of 2 antigens with the same serum: chicken ovalbumin (A_2), duck ovalbumin (A_1) and anti-chicken ovalbumin rabbit serum (B).

Sources of error: While carrying out reactions according to Ouchterlony's technique to obtain illustrations for this chapter, the author observed immobile false boundaries on several occasions. It is important to avoid anomalies of this sort since immobility is not a criterion for differentiation between false and true boundaries, as in the case of simple diffusion. The false boundaries observed were presumably caused by repeated refilling of the reservoirs, since they have not been observed in sealed Petri dishes. Formation of false boundaries is particularly likely if the 1st layer of agar fails to cover the curved portion of the bottom of the Petri dish sufficiently. However, they have been observed even when the surface of the 1st layer of agar was perfectly plane (Fig. 26).

In general, it would appear wise to avoid any procedure which might cause fluctuations in concentrations of reactants in the reservoirs such as refilling, or abrupt changes in temperature, although as yet, specific information is lacking with regard to the latter variable.

Identification.—The coalescence of zones provides a means for identification of antigens.§ If 2 antigens diffusing from parallel sources form zones of precipitate which coalesce completely, they may be considered identical; if the zones cross, the 2 antigens are different. Naturally, as long as the zones of precipitation fail to reach one another, we can conclude nothing. This may occur in the particular instance in which antibody concentration is very low and the concentrations of the antigens are very high, so that the 2 zones of precipitation are short, move quickly away from the sources of antigen and reach the antibody source before their extremities have united. Furthermore, Elek states that "lack of looping—and consequently erroneous interpretation—may occur if one of the lines is already well formed when a second approaches it" (8). Aside from this infrequent condition, the plates are usually easy to read if the number of zones of precipitation is not too great, especially when the antigens diffusing from the 2 sources are identical.

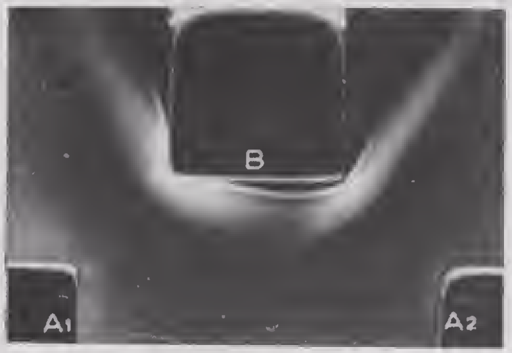

Fig. 26.—Artefacts due to repeated refillings of reservoirs. Reactants: ovalbumin at 2 different initial concentrations (A_1 and A_2) and homologous rabbit serum (B). Reservoirs were refilled at 1, 2, 3, 4 and 6 days after start of the experiment. Photograph taken on 7th day (33).

Identification can also be made using absorbed serum as Ouchterlony has done with diphtheria toxin (22). In this case it should be remembered that the density of the zones and the relative distances between them and the sources will vary with time. It is well to compare agar plates (or their photographs) before and after absorption, developed under identical conditions and for the same length of time.

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§ Or of antibody if an antigen is the reactant which diffuses from the perpendicular source.

TECHNIQUES APPLIED TO BACTERIOLOGY

Here we shall discuss the behavior of antigens diffusing into the gel from bacterial colonies growing upon it. Obviously the temperature, composition of the medium, etc., must be suitable for production of the antigens under investigation. Such techniques have been used from time to time since they were described by Sia and Chung (37) and Petrie (34) in 1932. The techniques may be considered either as simple diffusion or double diffusion in 2 dimensions.

Simple diffusion: Immune serum is incorporated in appropriate concentration with the agar medium which is then poured into Petri dishes.[‖] For methods of inoculation of the plates the reader is referred to the original articles (37, 34, 13, 35, 19–21).

It follows from the studies of simple diffusion that the zones of precipitation which may appear around a given bacterial colony will be less and less dense and more and more distant from the colony as the antibody content in the medium is decreased.

Double diffusion: Two similar techniques using agar in Petri dishes have been described by Ouchterlony (19–21, 25) and by Elek (7–9). Ouchterlony cuts out a transverse trench of agar and refills it with diphtheria antitoxin or antidiphtheria serum incorporated in agar. He then streaks 1 or more different strains of the diphtheria bacillus perpendicular to the trench. Elek allows a sterile strip of filter paper soaked in antitoxin to sink into the medium while the agar is still liquid. After the agar has hardened the organisms are streaked in parallel lines across the plate and perpendicular to the filter paper.

In both techniques zones of precipitation develop in a manner analogous to those discussed under double diffusion from perpendicular sources. The zones of precipitation commence at or near the point where the streaks of culture join the antitoxin source and form an acute angle with the streaks. If 2 zones of precipitation coming from neighboring streaks coalesce, it may be concluded that they are due to identical antigens; if the zones cross, the antigens must be different.

Various means may be used to identify which zone is due to precipitation of toxin itself. These include the time at which the zone appears, coalescence with a known zone from a known toxigenic strain or from a reservoir containing toxin, and dilution of the antitoxin so that zones caused by precipitation of nontoxic antigens will disappear.

[‖] This type of experiment has not been carried out in tubes. Perhaps it may prove of value in the case of anaerobic organisms.

Elek has applied the same technique to staphylococcus and *Clostridium welchii.*

All of these techniques, whether concerned with simple or with double diffusion, differ in 1 important respect from the case in which the antigens diffuse into the gel from a solution. In the latter, the antigen concentration will diminish continuously in the reservoir unless it is replenished. When antigen diffuses from a bacterial colony, the rate at which it is released is not known, regardless of whether its release is due to actual excretion of antigen or to bacterial autolysis. If one wishes to determine the number of antigens released, it must be kept in mind that variations in the concentration of antigen diffusing from the source may give rise to errors. Other sources of error must also be considered, particularly the possibility of nonspecific precipitate with constituents of serum, a possibility that necessitates the use of normal serum controls.

SPECIAL PROCEDURES FOR IDENTIFICATION

With each diffusion technique we have described general methods for identification of the individual zones of precipitation given by a mixture of antigens (see pages 356 and 373). It is also possible that information provided by determination of the diffusion coefficients (pp. 350 and 362) may be used to assist in identification. Finally, certain antigens endowed with enzymatic activity may be identified by means of visible reactions with a substrate incorporated in the gel (8). For example, Elek has cultivated *Cl. welchii* on agar plates containing human serum. When commercial serum was allowed to diffuse through the gel, Elek observed that the limit of opacity caused by α-toxin diffusing from the colonies (Nagler reaction) coincided with 1 of 2 zones of precipitation. He therefore identified this zone as being caused by the α-toxin. Elek also investigated staphylococcal hemolysin in an analogous manner.

It would seem, a priori, that any of the techniques of simple or double diffusion might be applied to the study of solutions of antigens with enzymatic activity as well as to those released from living bacterial cultures. In many cases it is not even necessary to incorporate the substrates in the gel until after the diffusion pattern has developed.

Cohn (5) has carried out an experiment of this type using the β-galactosidase of *Escherichia coli* as antigen with its homologous rabbit antiserum. The double diffusion technique of Ouchterlony was followed. After 6 days, the substrate, o-nitrophenyl β-galactoside (NPG) was sprayed over the agar surface. An intense yellow color was produced by hydrolysis of NPG in the region of antigen excess which was limited by the zone of precipitation.

This method of identification can be further extended for general use when the product of enzyme action, though not itself visible, can be rendered visible at a convenient time by addition of a suitable indicator.

In using diffusion methods for identifying enzyme-antienzyme systems, many factors will require consideration. These will vary with each system investigated and will depend on: the diffusibility of the reaction products, whether or not enzyme activity is inhibited by specific antibody, the time when the substrate and perhaps indicator are added to the medium, whether the initial concentration of antigenic enzyme is in excess relative to antienzyme or vice versa, etc., etc.

It should be stressed that if antibody (antienzyme) is in excess, the limit of enzyme-substrate reaction may not necessarily coincide with the antigen-antibody equivalence point and consequently may not coincide with the zone of precipitation in a flocculation-type reaction.

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